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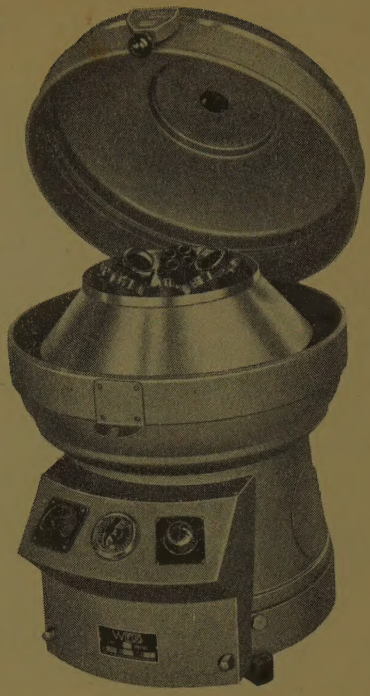

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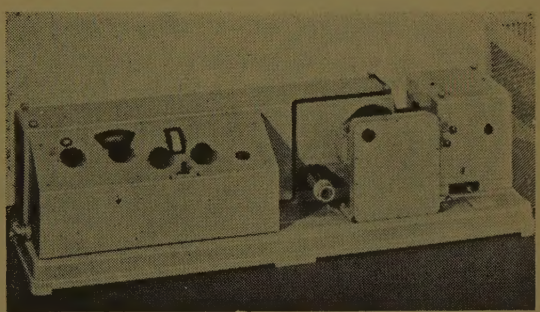

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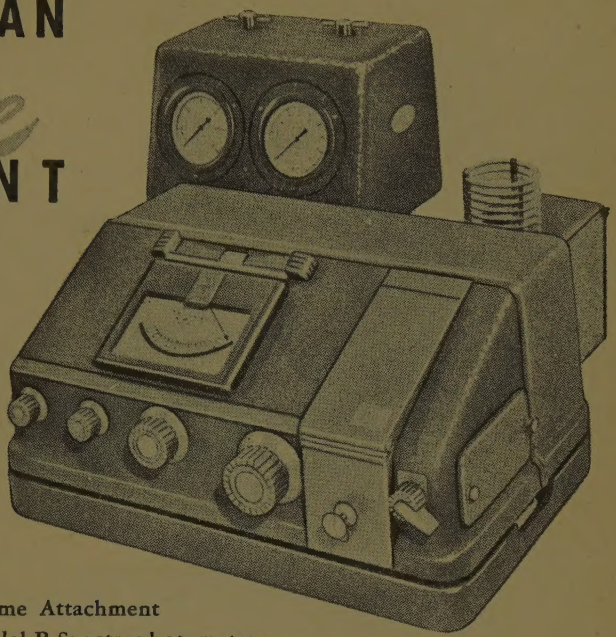

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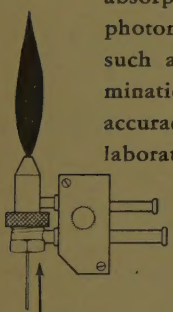

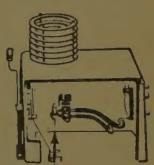

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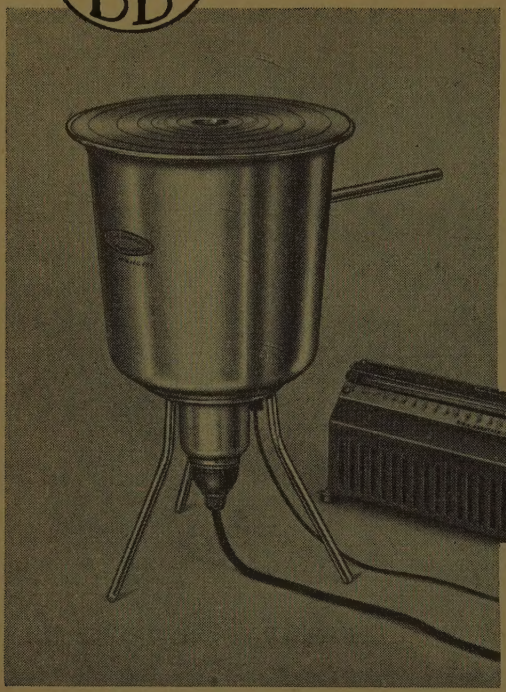

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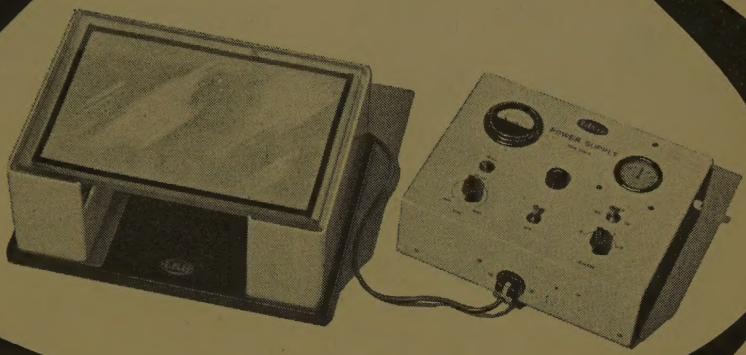

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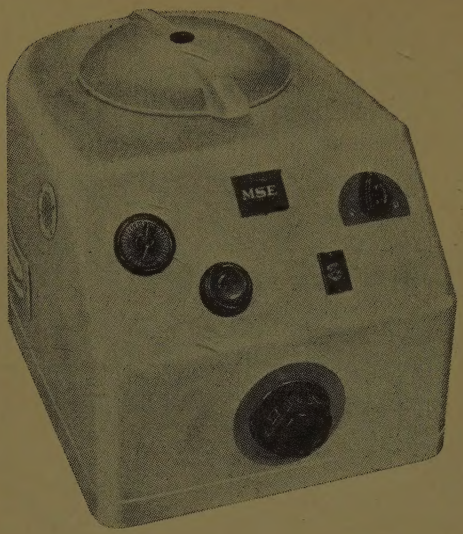

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PAPER ELECTROPHORESIS OF ISOLATED PLASMA PROTEIN FRACTIONS

By S. CHR. SOMMERFELT[1]

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(Received for publication April 10, 1953)

Since the first articles on paper electrophoresis of proteins appeared in 1950 (Turba & Enenkel, Cremer & Tiselius, Durrum, Grassmann & Hannig), the method has rapidly become a common one because it is simple and affords valuable information concerning the serum proteins. The following is an attempt to elucidate in part the basis of the method by investigating the relationship between the amount of protein applied and the measurable color intensity. By subjecting varying amounts of isolated serum or plasma protein fractions to ordinary paper electrophoresis, it is possible to determine whether this relationship, when represented graphically, follows a straight line or some other curve, and whether this curve is the same for the different fractions. The results of such investigations will differ according to the electrophoretic method employed. Of particular significance are the dye employed, and whether elution or direct photometry of the strip is performed (Sommerfelt, 1953).

METHOD AND TEST SOLUTIONS

Grassmann & Hannig's method (1952) is employed in the present investigation with certain modifications (Sommerfelt, to be published). The principle involved is migration on freely suspended, thin paper, staining with amidoschwarz 10 B (Bayer) and direct photometry of the strip.

Bovine plasma protein solutions. 2.5, 1.0, 0.5 and 0.2 per cent solutions prepared from crystallized bovine plasma albumin (Armour) and from bovine γ-globulin (Armour).

The solutions were controlled by micro-Kjeldahl analysis according to Hiller, Plazin & van Slyke (1948), and compared with known solutions of ammonium sulphate for control. The calculation factor employed was 6.25. The results showed that the solutions were slightly weaker than calculated. They were stored in a refrigerator and remained

[1] Present address: Drammen Hospital, Drammen, Norway.

clear with no change in the result of the nitrogen analysis for 22 days (albumin) and for 15 days (γ-globulin). They were not controlled for a longer period.

Human serum protein fractions. Albumin, α_2-, B- and γ-globulin were prepared in pure form by means of paper electrophoresis, and the protein concentration of the solutions was determined by the biuret method according to Levin & Brauer (1951).

RESULTS

Bovine albumin. Varying amounts of the different dilutions of bovine albumin were subjected to electrophoresis, staining and recording in the usual manner. Curve A in Fig.1 shows the results of planimetry plotted against the amount of protein. It is striking that the curve does not pass through origo. The deviation is indeed slight, but nevertheless significant. The most probable explanation would seem to be the following: During the electrophoretic migration, some of the albumin adheres to the filter paper,

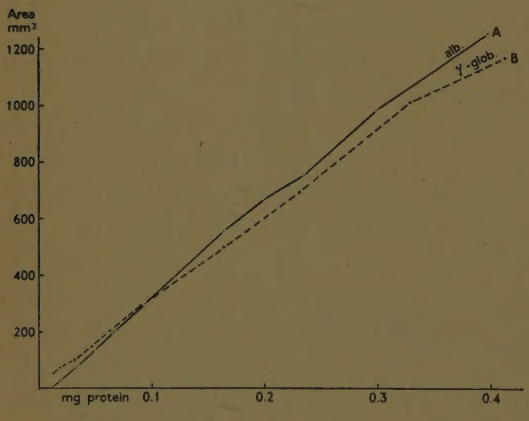


Fig. 1. Bovine plasma albumin (A) and γ-globulin (B). Ordinary technique. Each point indicates the mean of 2—4 analyses.

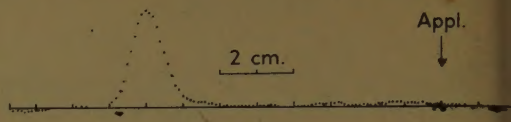


Fig. 2. Bovine plasma albumin. 0.010 ml of 0.9 per cent solution. 14 hours — 110 Volts.

and this escapes the measuring of the area of the albumin peak.

The individual electrophoresis curves (Fig. 2) seem to exhibit the same phenomenon: there is, although very slight, an elevation of the curve from the site of application toward the base of the peak. This elevation seems to be independent of the amount of protein applied, i. e. the paper adsorbs a certain quantity of albumin and is then saturated. According to curve A in Fig. 1, the quantity of albumin lost should be 0.010—0.015 mg, corresponding to an area of about 50 mm². The area between the elevated curve and the base line is of approximately this magnitude, but shows considerable variation in different experiments.

The question of adsorbtion and its approximate magnitude can be approached in other ways. If the albumin was applied in the usual manner and then dried and stained in the usual way, without allowing any migration to take place, nothing of the protein would be lost through adhesion outside the protein stripe. This type of stripe, however, was very narrow and sharply delineated, and the curve obtained by direct photometry was much higher and steeper than an ordinary curve after migration. It was, therefore, desirable to try to produce albumin stripes which had not migrated, or a very short distance only, but which resembled the ordinary ones both in breadth and limitation. This

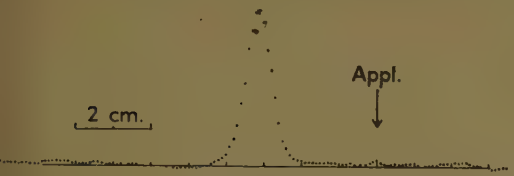


Fig. 3. Bovine plasma albumin. 0.010 ml of 0.94 per cent solution. 6 hours — 70 Volts.

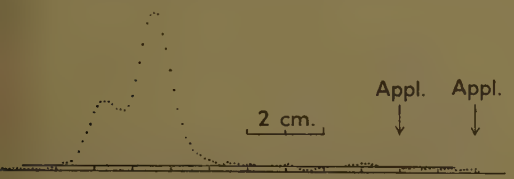


Fig. 4. Bovine plasma albumin. 0.007 ml of 0.94 per cent solution in 17½ hours. 0.010 ml of 0.94 per cent solution in 13½ hours. 110 Volts.

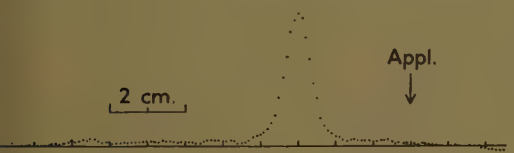


Fig. 5. Bovine plasma albumin. 0.010 ml of 1.14 per cent solution in 13 hours to the left, thereafter 11 hours to the right. 110 Volts.

was achieved by using a short migration time and low voltage (5—6 hours, 50—70 V) as shown in Fig. 3. The results of planimetry of such curves are illustrated by curve B in Fig. 6. It will cut the abscissa near origo. Here the albumin has migrated such a short distance that very little is lost by adsorbtion to the paper. The fact that the curve is practically parallel to curve A in Fig. 6 (= curve A in Fig. 1), also indicates that the adsorbtion is independent of the quantity of albumin applied.

Further: A certain amount of albumin was applied and the migration was begun. After 4—6 hours a new amount of albumin

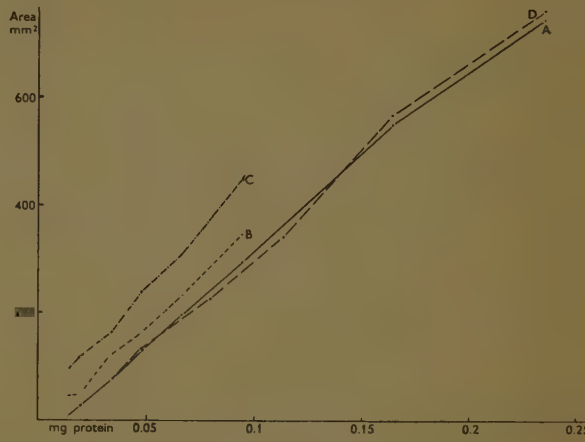


Fig. 6. Bovine plasma albumin. A: ordinary technique (= A in Fig. 1); B: 6 hours — 70 Volts; C: after preceding saturation of the paper with albumin; D: migration forwards and backwards. Each point in B, C, and D indicates the result of a single analyses.

was applied to a part of the paper strip where the first portion of albumin had passed (1—2 cm from the starting point). From this point the migration was allowed to continue for the usual period of time. Photometry gave a curve like that shown in Fig. 4. Curve C in Fig. 6 shows the results for various amounts of protein. This curve goes almost to origo, indicating that nothing of the second portion of protein is lost by adsorbtion.

When the albumin was allowed to migrate the ordinary distance and then, by switching the poles, to migrate back again practically to the starting line, a curve like that shown in Fig. 5 was obtained. The elevation extends as far as one might assume that the albumin has migrated, and is of the same order of size as in Fig. 2. Planimetry gives the results shown in curve D, Fig. 6. This curve almost

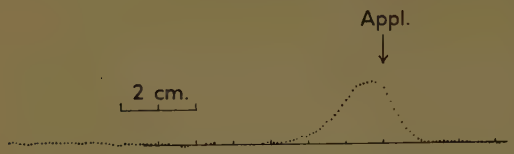


Fig. 7. Bovine plasma γ-globulin. 0.010 ml of 0.90 per cent solution. Ordinary technique.

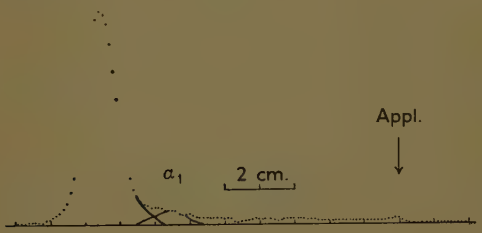


Fig. 8. Human serum albumin. 0.011 ml of 1.75 per cent solution. Ordinary technique.

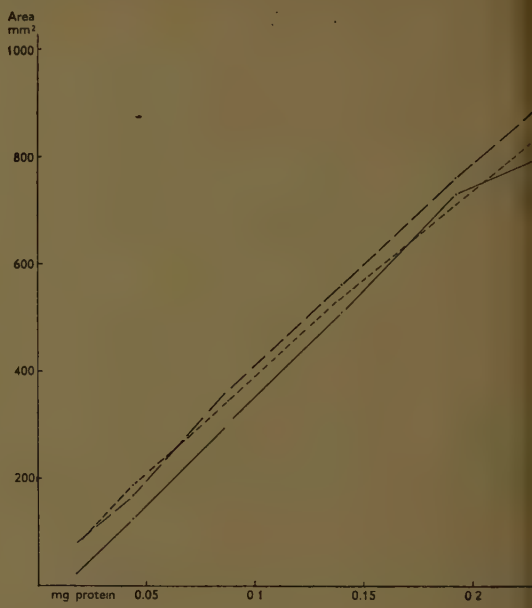


Fig. 9.

Fig. 9—12. Human serum protein fractions. A ordinary technique; B: 6 hours — 50 Volts; C after saturation of the paper with albumin. Eac point indicates the result of a single analysi Fig. 9: Albumin. Fig. 10: α_2-globulin. Fig. 11 β-globulin. Fig. 12: γ-globulin.

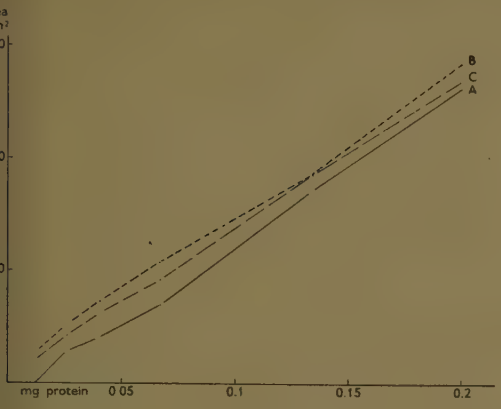
coincides with A in the same figure. It thus appears as if the paper adsorbs a certain amount of albumin and then becomes saturated.

Bovine γ-globulin. Various amounts of the different dilutions of bovine γ-globulin were subjected to ordinary electrophoresis. Fig. 7 shows a typical curve, and the results are presented in curve B, Fig. 1. This curve passes through origo. With the method employed the γ-globulin spreads out from the starting point and in this way there is no loss by adsorbtion.

Mixtures of bovine albumin and γ-globulin were also investigated. The results obtained practically coincide with curves A and B in Fig. 1, with a certain tendency for γ-globulin to lie a little higher than B, in agreement with the fact that it has acquired a slight addition of adsorbed albumin.

Human albumin. This is often contaminated with α_1-globulin, Fig. 8. Determination by the biuret method will include all of th protein in the solution, and the α_1-area i therefore included in the albumin value This will not lead to any significant erro

Fig. 9 shows the results when varyin amounts of albumin are subjected to pape electrophoresis by the usual method (curv A), for a short period with low voltag (curve B) and electrophoresis on a pape where another albumin solution had migrate to saturate the paper (curve C). Here als it is striking that A cuts the abscissa at 0.01 —0.015 mg, corresponding to the approx mate amount adsorbed to the paper, while th



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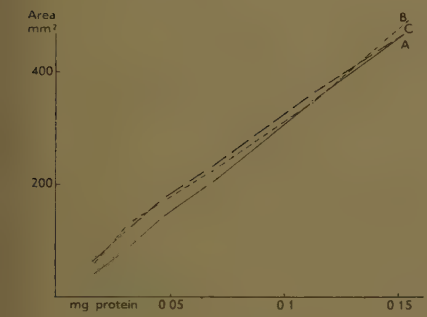

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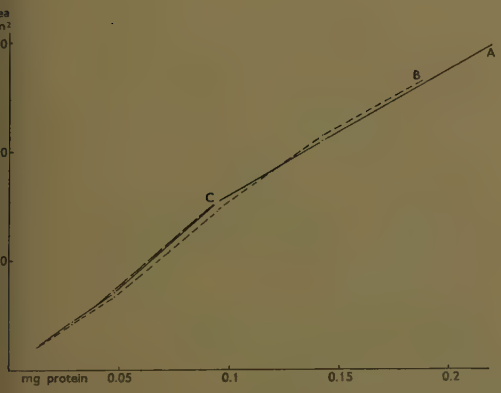

Fig. 12.

two other curves are decidedly higher and go almost to origo.

Human globulin fractions. In Fig. 10 the curves for α_2-globulin exhibit the same characteristics as those for albumin.

The same is true of the curves for β-globulin, Fig. 11, but here there is less difference between the curves, since β-globulin migrates a shorter distance than albumin and α_2-globulin and therefore loses less by adsorbtion.

Further, the curves in Figs. 10 and 11 seem to show that when the paper is saturated with one protein, namely albumin (the C curves), it will no longer adsorb detectible quantities of the other serum proteins. The individual elctrophoresis curves give the impression that a certain amount of adsorbtion occurs (see Fig. 14), but it is very slight compared with the amount adsorbed on untreated paper.

Fig. 12 shows the curves for γ-globulin. As expected, there is no noticeable difference between the curves. There is no loss by adsorbtion of γ-globulin, as the compound spreads out from the site of application with the method employed.

DISCUSSION

It must be emphasized that the paper electrophoresis method for fractionation of proteins exhibits considerable variation. An investigation of the reproducibility of the method by serum analyses (Sommerfelt, 1952) showed a standard deviation of 5—10 per cent for the small globulin fractions. If the range of variation is set at $\pm$ 2—3 times the standard deviation, this will give a variation of $\pm$ 10—30 per cent, expressed in per cent of the individual fractions. Other

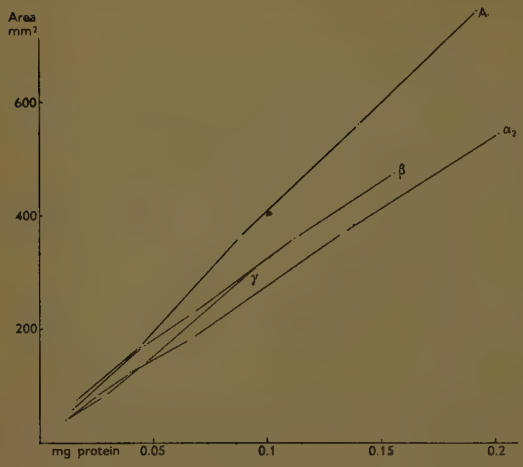

Fig. 13. Human serum protein fractions. After preceding saturation of the paper with albumin (= C-curves in Figs. 9—12).

investigators, some using different methods, have obtained similar values (Flynn & de Mayo 1951), although many (Grassmann & Hannig 1952, Goa 1952) express the standard deviation in per cent of the total protein content, whereby the actual figures will be much smaller and may be misleading.

In serum analyses the albumin will migrate in front. Some of the albumin will be adsorbed to the paper. The globulins, on the other hand, migrate on paper saturated with albumin and will not lose significant amounts by adsorbtion. On the contrary, the globulins will gain a small quantity of the adsorbed albumin, and thus their values will be slightly raised. To avoid this, the paper could be saturated with albumin beforehand as in the C curves in Figs. 9—12. Fig. 13 shows a composite of these curves. All of them pass almost through origo but upward they exhibit considerable divergence. When planimetry is performed and the serum

Fig. 14. Human serum protein fractions. 0.005 m of 1.75 per cent albumin solution in 19 hour 0.010 ml of 0.67 per cent α_2-globulin solution in hours. 110 Volts.

electrophoresis curve is calculated, it assumed that the same quantities of th various protein fractions yield approximatel the same areas, i. e. that curves like thos in Fig. 13 will coincide. This cannot achieved by first treating the paper wit albumin.

On untreated paper the albumin will adsorbed and different quantities of albumi will give the curve A in Fig. 9. The globulin will then migrate on paper saturated wit albumin. Fig. 14 shows an example for α_2 globulin. Planimetry with bl_1 as a base lin will give the area which actually correspond to α_2. In serum electrophoresis, howeve the base line will be drawn on the basis the points outside of the albumin and ou side of the site of application, i. e. bl_2 Fig. 14. α_2 will thus have an additio namely part of the adsorbed albumin. A cording to Fig. 14 this addition should quite large due to the considerable breadt of the base of α_2. In serum electrophoresi however, there will be a certain amount overlapping of the Gaussian curves for th globulin fractions. In accordance with th they will have to share some of the additi due to albumin, and consequently this add tion will be somewhat reduced for the indiv dual fractions. By drawing an assumed limit tion (the perpendicular lines in Fig. 14) towa

ıe neighboring fractions, an approximation
f the magnitude of this addition may be ob-
ined from the curves. If the individual
ectrophoresis curves are measured by plani-
etry with this addition, and the results are
otted against the amount of protein, Fig. 15
obtained. These curves should correspond
pproximately to the situation in serum
ectrophoresis.

The albumin curve deviates considerably
its lower portion from the globulin curves.
approaches them, however, above, cuts
ıem and bends off in about the same direc-
on as the globulin curves. In serum electro-
ıoresis the albumin values obtained will
sually lie in the upper part of the given
ırve, and even with a considerable albumin
eduction it should not be necessary to go
elow 0.080—0.100 mg albumin, as the quan-
ty of serum applied may be increased. Of
ıe globulin curves, that for α_2 deviates from
ıe β and γ curves. The latter two curves
ın very close in their lower portions and
ere they have almost the same direction as
ıe most significant part of the albumin
ırve.

A comparison between the curves in Figs.
3 and 15 seems to indicate that it is
st not to treat the paper with albumin
eforehand, since the adsorbtion of albumin
as a certain compensatory effect on the
reater stainability of this protein.

Theoretically, the most satisfactory pro-
dure would be to set up standard curves
r the individual protein fractions (as in
ig. 15) and then to read mg protein for the
fferent areas found by planimetry of the
rum electrophoresis curves. It is, however,
ubtful whether there is much to be gained
this procedure, because the method itself

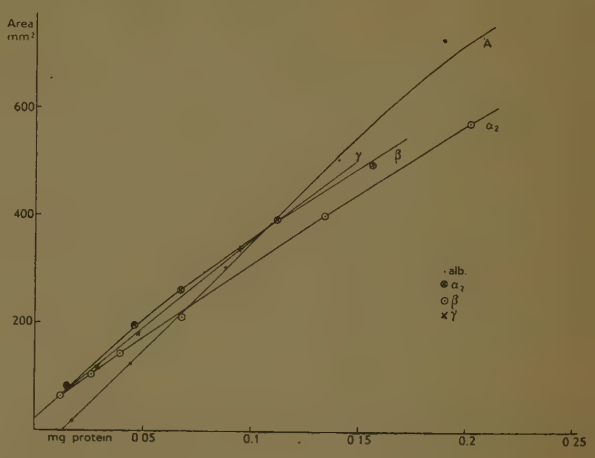

Fig. 15. Human serum protein fractions measured with the planimeter under conditions similar to those under which ordinary serum electrophoresis curves are measured. (See text.)

has such wide variation. Here it should also be pointed out that it may well be that the stainability of the individual fractions varies from one serum to another, especially under pathological conditions. The work of Köiw, Wallenius and Grönwall (1952) seems to indicate that this is true of bromphenol blue.

Grassmann & Hannig (1952) have also performed investigations with pure protein fractions. They moistened the filter paper with protein solutions of different strengths and measured the extinction with a recording apparatus. The curves of the results for albumin and γ-globulin had a linear course in their lower portions, but curved slightly upward, especially γ. The curve for γ was decidedly below that for albumin, indicating that albumin had a higher stainability.

After paper electrophoresis of various quantities of albumin and globulins, these authors set up standard curves. There was

little difference between the albumin and γ-globulin curves, and the authors explain this as due to the difference in form of the albumin peak and the γ peak, as the extinction for a high, narrow peak (albumin) will be somewhat reduced by photometry in the recording apparatus compared to a lower and broader peak (γ). This is perhaps significant when dealing with larger amounts of protein, but hardly with the small amounts dealt with in practice. Here it must be assumed that the above-mentioned adsorbtion phenomenon is of greater significance as a corrective factor. Grassmann & Hannig do not mention this and their albumin curve passes through origo, but their standard of measure is a small one, so this may be the reason why the phenomenon is not manifest.

CONCLUSION

The present investigation seems to show that there is a satisfactory basis for using the dye amidoschwarz 10 B (Bayer) and direct photometry of the stained strip in paper electrophoresis analysis of serum proteins. It is true that analyses of the same amount of isolated serum protein fractions do not yield equally large areas for all the fractions, but if the results are set up in curves with the amount of protein as the abscissa and the area of the peaks as the ordinate (Fig. 15), it is seen that the most significant parts of the curves coincide fairly well. The deviations can hardly be very important since the variations of the method on the whole are quite wide.

During the electrophoretic migration a certain amount of protein is adsorbed to the filter paper. Thus, paper electrophoresis does not give a complete separation of different protein fractions, as it is often stated in the literature. By serum electrophoresis the globulin fractions always include a small amount of adsorbed albumin.

SUMMARY

The purpose of the present investigation has been to examine in more detail a part of the basis for paper electrophoresis, namely the relationship between the amount of protein applied and the measurable color intensity. Bovine albumin and γ-globulin, as well as human albumin, α_2-, β- and γ-globulin were investigated by Grassmann & Hannig's (1952) method, with some modifications (Sommerfelt, to be published). The principle involved is migration on freely suspended, thin paper, staining with amidoschwarz 10 B (Bayer) and direct photometry of the strip.

1. There is good agreement between the results with the bovine and human preparations.

2. Albumin shows the greatest stainability of the fractions investigated, α_2-globulin the least.

3. During electrophoretic migration an adsorbtion of protein to the paper takes place.

4. The amount of protein adsorbed is independent of the amount applied, i. e. the paper becomes saturated and then adsorbs no more of the same protein.

5. When the paper is saturated with albumin, only a very slight adsorption of other fractions occurs.

6. In electrophoresis of serum, the adsorbtion causes a reduction of the albumin and a slight increase of the globulin values

thus compensates, to some extent, the eater stainability of the albumin.

7. The present investigation seems to in-cate that there is a satisfactory basis for nploying the described electrophoretic ethod in studying serum proteins, but the nsiderable variation of the method must be nphasized.

ACKNOWLEDGEMENTS

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AN ELECTROMETRIC METHOD FOR THE DETERMINATION OF CHOLINESTERASE ACTIVITY

III. CHOLINESTERASE ACTIVITY IN BLOOD SPOTS ON FILTER PAPER

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Michel's method for electrometric determination of cholinesterase in human blood (Michel 1949) and especially its modification by Tammelin (Tammelin 1953) are accurate and handy enough to suit clinical and research work. However, vein puncture is inconvenient for the patient and centrifugation for separating serum and erythrocytes is rather timeconsuming. Moreover the cholinesterases in blood samples are unstable (Strehler & Meyer 1952). The use of a refrigerator is necessary and hemolysis may occur in the fresh blood sample. On the suggestion of Dr. Davies (personal communication) filter paper was used for keeping the blood samples. The principal advantage of this method is the use of blood from finger tip instead of venous blood and the stability of the enzyme in dried paper spots.

Apparatus

The same apparatus as described by Tammelin(1953) is used. However, some smaller modifications had to be made. It was found by eluation experiments that only cholinesterase is eluated from the paper, while about 80 per cent of the acetylcholinesterase is fixed to paper. For this reason the determinations must be made directly on the pieces of paper. This caused disturbances due to lack of circulation of the surrounding solution. However, results were reproducible, if a slight shaking of the electrode vessels was maintained.

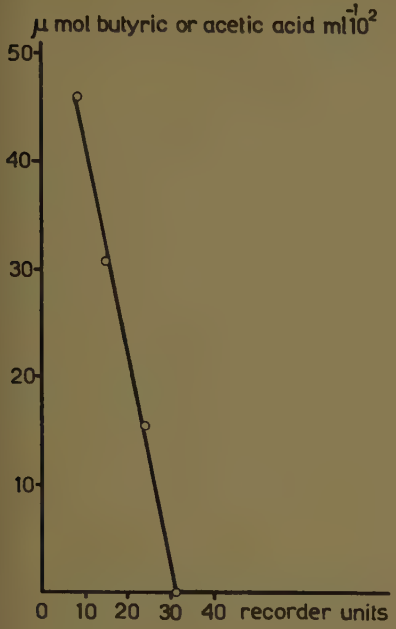
As this method works with very small quantities of blood (about 0.01 ml blood per ml final solution) the sensitivity of the aforementioned apparatus had to be increased. Thus a bigger part of the potential generated by the pH-meter was allowed to operate upon the recorder by changing the potentiometer between these instruments.

Calibration of the apparatus

The calibration of the apparatus is performed as usual. 1.5 ml buffer, 2 paper discs with enzyme and 1.76 ml. aq. redest. are used. Known quantities of butyric acid or acetic acid were introduced in the vessels. The two acids give the same inclination of the lines as could be expected from their close dissociation constants. The calibration diagram, which also establishes the sensitivity of the apparatus, is shown in Fig.

Solutions

The buffer capacity of Michel's buffer is too high for this purpose. However, dilution of the buffer 1 : 1 with aq. redest. gives higher sensitivity without loss of the linear relationship betwee



g. 1. Calibration diagram for conversion of re-rder units to μmol butyric or acetic acid ml^{-1}.

zyme concentration and measured activity. The ffer used has the following composition:

4.123 g (0.02 mol) sodium barbital
0.5446 g (0.04 mol) KH_2PO_4
44.730 g (0.6 mol) KCl

uted to about 1900 ml with aq. redest., 28 ml of .00 M HCl is added and the volume made up to 00 ml. According to Augustinsson (in press) two ferent substrates were used for determination of olinesterase and acetylcholinesterase. There is need to separate the two types of cholinesterases, only acetylcholinesterase splits acetyl β-methyl-oline and only cholinesterase can split butyryl-oline. (Augustinsson 1949). The substrate solu-ns have the following compositions: butyryl-oline bromide 0.249 g in 5 ml aq. redest. acetyl nethylcholine iodide (mecholyl) 0.281 g in 5 ml redest.

Source of enzyme

With a lancet a puncture is made in the ıger tip and the blood droplets are allowed to fall on a piece of filter paper. The paper must absorb the blood easily. Munktell No. 8 was found to be satisfactory. The paper sheet should be held at a slight angle, thus helping the blood to spread out properly. For duplicate determinations of both acetylcholinesterase (main part in erythrocytes) and cholinesterase (main part in serum) activity, 4—8 spots (depending on size) are necessary. They are easily obtained from one puncture. The spots are allowed to dry. Two discs having a diameter of 1 cm are punched for each determination. They are cut into three pieces and placed in an electrode vessel having a diameter of 2 cm.

METHOD OF DETERMINATION

Duplicate determinations are always performed. Each electrode vessel contains:

2 punched discs
(as acetyl β-methylcholine splits very slowly, 3 discs were preferred for determinations of acetylcholinesterase)
1.5 ml buffer solution
1.5 ml aq.redest.

The electric equipment is connected and the electrodes allowed to reach equilibrium with the solution. This takes at least 30 minutes. 0.3 ml of the substrate solution are added, giving a final concentration of $1.8 \cdot 10^{-2}$ M for butyrycholine and the same for acetyl β-methylcholine. The experimental results are treated as described earlier (Tammelin 1953).

RESULTS

To make sure that no difference between the cholinesterase activity in venous and capillary blood can be found, identical determinations of the two kinds of blood were performed. No difference could be observed.

Determinations were made to establish the uniform absorption of blood in filter paper. Forty discs, twenty with and twenty without blood, were weighed. Thus the blood

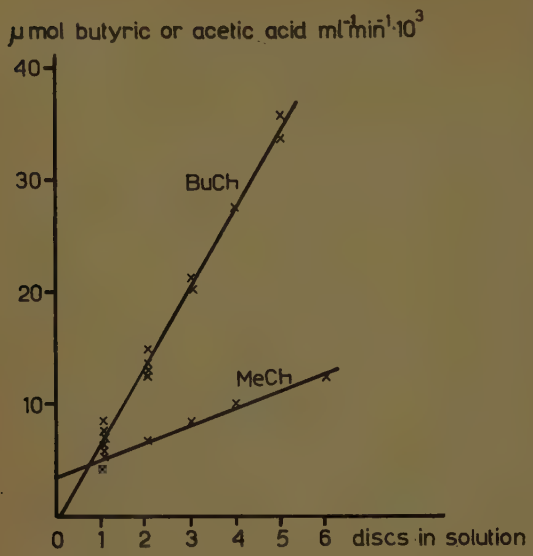


Fig. 2. Diagram showing enzyme activity as function of the number of blood paper discs.
BuCh = butyrylcholine bromide.
MeCh = acetyl β-methylcholine iodide.

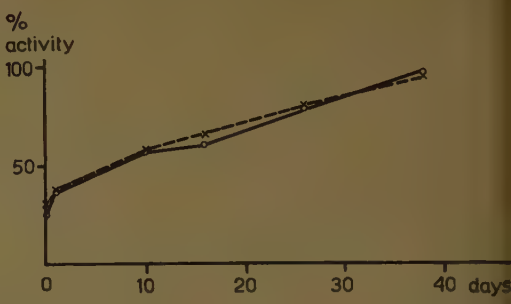


Fig. 3. Diagram showing recovery of cholinestera in dogs after intoxication.
— — — — — = conventional method.
———— = paper spot method.

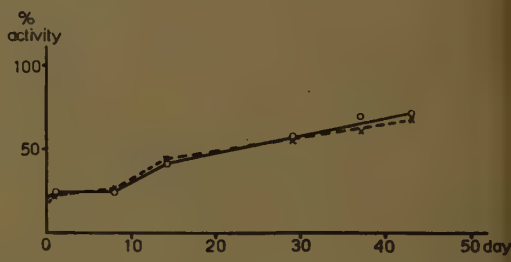


Fig. 4. Diagram showing recovery of acetylcholi esterase in dogs after intoxication.
— — — — — = conventional method.
———— = paper spot method.

weight in one disc was found to be 3.66 mg ± 0.12. The standard deviation is 3.3 per cent. This shows that it is unnecessary to use a pipette or to weigh the discs. However, care should be taken to avoid dropping the blood near the paper's edge.

Fig. 2 shows that the enzyme activity obtained is proportional to the number of paper discs, at least within the limits of 6 discs.

The results obtained with discs were compared to results obtained by introducing blood directly into the elcetrode vessels. 0.05 ml of blood was dropped with an Ellerman pipette on filter paper and in the vessels. No difference in activity was found.

The recovery of cholinesterase activity and acetylcholinesterase activity in dogs intoxicated by a cholinesterase inhibitor was followed by the two methods. The resu are shown in Figs. 3 and 4.

The use of the paper spot method determination of I_{50} values for choli esterase inhibitors was demonstrated wi Tabun. The pI_{50} was found to be 7.64. T conventional electrometric method gives 8. (Lundin, personal communication).

It was found that the stability of cholinesterases in paper spots considera exceeded that in ordinary blood samples. a rule the blood spots used were about weeks old. However, spots stored at ro temperature still showed the same activ 5 months later.

DISCUSSION

The method described seems to be well plicable in cases where series of cholin- erase determinations are necessary or here the blood tests have to be transported. he advantages of the method often counter- lance the disadvantages of the slightly duced accuracy and the longer time neces- ry for obtaining equilibrium at the elec- odes.

ACKNOWLEDGEMENT

My sincere thanks are due to the head of this stitute, Professor G. Ljunggren, for the kind erest he has shown in this work.

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SERUM IRON, TOTAL IRON-BINDING CAPACITY OF SERUM, AND SERUM COPPER IN NORMALS

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(Received for publication April 10, 1953)

The presence of iron in serum was first established thirty years ago. Until then the small quantities of iron demonstrated in serum had been supposed to originate from hemoglobin and to admix by slight hemolysis. Fontès & Thivolle (1925) determined the serum iron concentration in horses and found it reduced in cases of iron deficiency. A few years later O. Warburg (1927) made rather exact serum iron determinations by a catalytic method. Various other determinations have since been carried out. In spite of the different methods employed the mean values in normals vary but little (for literature see Andersson, 1950). All the workers found the highest serum iron values in males, most often with a rather considerable sex difference.

Present investigations

Blood was withdrawn after brief stasis with cannulae of chrome steel without stilette. I have been unable to procure internally polished cannula, but have had occasion to compare a sample taken with a chrome steel cannula with one taken simultaneously with an internally polished cannula. The values were 58.8 μg per cent and 59.3 μg per cent respectively (average of 4 duplicate determinations). This difference is not greater than might be expected according to the accuracy of the method. The method is that indicated by Brøchner-Mortensen (1940) with a few modifications.

Furthermore I have compared Brøchner-Mortensen's method and the phenanthroli method on one sample of serum and four the same results with both methods.

After addition of hydrochloric acid t serum was left for one hour at 37° C. T trichloracetic acid was added, and aft standing in 10 minutes the mixture w centrifuged for 20 minutes. Reading w performed in a 5 cm semimicrocuvette. was thereby possible to determine serum ir and serum copper in 3 ml of serum, a serum iron in 3 ml centrifugate. (See al determination of serum copper.)

Unlike Brøchner-Mortensen (1940), w removed the precipitated protein by filtr tion, I used centrifugation. This procedu will always give a clear centrifugate. Centr fugation has the advantages that more centr fugate than filtrate is obtained, the troub of procuring iron-free filters is avoide and the glassware is more easily cleaned, no glass funnels are required.

Brøchner-Mortensen (1940) showed th with his method the serum iron determin tion is influenced but little by hemolysis the serum used. As I left the serum samp for one hour at 37° C, instead of for minutes at room temperature, larger amoun of iron might be liberated from the hem globin by a possible hemolysis in the seru

I studied the influence of hemolysis in the llowing way. To 2 ml of serum I added 1 ml of redistilled water, in which different amounts of blood were hemolysed.

Iron concentration in 2 ml serum + 1 ml water = 118 μg per cent.
Iron concentration in 2 ml serum + 1 ml water + 0.01 ml blood = 119 μg per cent.
Iron concentration in 2 ml serum + 1 ml water + 0.025 ml blood = 121 μg per cent.
Iron concentration in 2 ml serum + 1 ml water + 0.1 ml blood = 143 μg per cent.

As, thus, a considerable hemolysis is quired to raise the serum iron value, a emolysis that is not directly observable ust be supposed to be of no importance for e serum iron determination.

Analysis of solutions with a known iron ncentration: Iron concentration 100 μg er cent. Extinction (mean): 0.264. Con-ersion factor: 379. (Extinction × 379 = μg er cent Fe in serum.)

The color reaction was in accordance with eer's law, and added iron solutions were covered quantitatively.

Serum iron was determined in 40 normal omen and 40 normal men. The test samples ere withdrawn at 9 a.m. Duplicate deter-inations were made throughout. Hb per nt and E.S.R. were examined and found ormal. The men were from 14 to 40 years d, 21 under 30 and 3 were 40. The women ere from 16 to 62 years old, 20 were under) and 8 over 40.

The mean value for the men was 124.7 g per cent with a standard deviation of the dividual observations of ± 25.3 μg per nt, and for the women 101.1 μg per cent ith a standard deviation of the individual bservations of ± 24.4 μg per cent. The stribution follows approximately the bi-omial law. The difference between the ean value for men and women is statistic-ly significant.

The percentage deviations of duplicate determinations followed the binomial law of distribution. The estimate of the standard error of the individual observations was calculated at ± 2.4 per cent, and the standard error of the duplicate determinations at $\frac{\pm 2.4}{\sqrt{2}}$ per cent. The percentage deviation does not depend on the serum iron concentration.

DETERMINATION OF THE TOTAL IRON-BINDING CAPACITY OF NORMALS

Previous investigations

Laurell (1947) ascertained that iron added to serum will at first be bound in the same way as the natural serum iron. Serum possesses, in other words, an iron-binding capacity. This iron-binding capacity added to the serum iron constitutes the total iron-binding capacity of serum. This varies characteristically in different diseases.

Laurell (1947) determined the total iron-binding capacity by saturating the transferrin in serum (the iron-binding protein) with iron added in excess to the serum. The excess iron was bound by adding phenanthroline. Addition of acid caused liberation of the protein-bound iron, while the phenanthroline-bound iron was precipitated and could be filtered together with the protein. The iron concentration of the filtrate was

Table I. *Total iron-binding capacity of serum in normals.*

Year	Author	Number of patients	Sex	Mean	Min.	Max.	Number of patients	Sex	Mean	Min.	Max
1947	Laurell	61	♂	315 µg%	254	406	39	♀	315 µg%	245	395
1949	Rath et al.	15	»	311 »	254	432	15	»	288 »	224	415
1949	Cartwright et al. .	15	»	347 »	306	396	15	»	371 »	316	429
1951	Ventura et al. ...						25	»	328 »	241	439

Survey of the most important determinations of the total iron-binding capacity in normals.

determined in the usual way. Rath & Finch (1949) and Cartwright & Wintrobe (1949) determined the unsaturated iron-binding capacity by means of the salmon-red color of the iron-binding protein in iron-saturated condition. Table I shows the results of determinations of the total iron-binding capacity of serum in normals.

Present investigations

Laurell's (1947) method C was employed to determine the total iron-binding capacity of serum. Minor alterations were made owing to the difficulty of procuring suitable chemicals

METHOD

Reagents:

Hydrochloric acid for analysis, 6-normal.

Trichloracetic acid for analysis 20 per cent.

Ammonia water for analysis, concentrated.

n/2 hydrochloric acid for analysis.

Paradinitrophenol for analysis 1 per cent solution in absolute alcohol.

Phenanthroline 2 per cent solution in 10 per cent alcohol.

Sodium hydrosulphite ($Na_2S_2O_4$) for analysis.

Ferriammonium sulphate solution (100 mg Fe and 1 ml HCl per litre) with 2 ‰ ascorbic acid added immediately before use.

Hydrochinone for analysis 4 per cent solution.

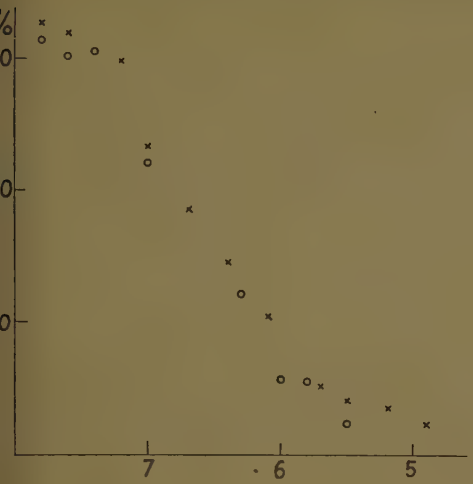
PROCEDURE

To two serum samples of 1 ml I added 0.05 ml of ferro-iron solution plus 0.025 ml redistilled water or 0.075 ml of ferro-iron solution. After 5 minutes 0.05 ml of phenanthroline solution and 1.5 mg sodium hydrosulphite were added to each sample which were then left for one hour. 1 ml of the mixture was now dripped, under cautious shaking into a test tube containing ¾ ml of 6-normal hydrochloric acid and 0.5 ml of redistilled water. After 10 minutes 1.5 ml of trichloracetic acid were added and the mixture shaken. Filtration was performed after another 10 minutes. To 1.8 ml of filtrate in a graduated flask holding 5 ml were added first 2 drops of paradinitrophenol solution and next concentrated ammonia water till yellow color was attained. The mixture was now titrated with n/2 hydrochloric acid to change of color again. 2 drops of concentrated hydrochinone solution and 0.1 ml of 2 per cent phenanthroline solution was now added. The flask was filled up to the mark with redistilled water and a thorough mixing followed. After 30 minutes reading was performed in a Pulfrich stufen-photometer with filter S_{50} and 5 cm semi microcuvette against a blind.

Laurell's method has not been employed by any other workers. It was therefore necessary to test the various stages.

1. *Influence of pH variation on determination*

Fig. 1 shows the total iron-binding capacity in two different serum samples, when addition of n/2 or n/10 hydrochloric acid to



. 1. Determinations of the total iron-binding acity of serum at different pH-values. Ordinate: total iron-binding capacity in μg per cent, abscissa: pH.

ops had made the pH fall even to 4.9. e curve runs a horisontal course within pH range of 7.4 to 7.8, where the iron- ding capacity of serum was determined, shows rapidly falling values on the acid e of this, the transferrin being no longer urated with iron.

According to Vahlquist (1941), serum n is first dialysable at pH 5. In the pre- t experiment the iron was liberated at a siderable more alkaline pH. This was sumably due to the fact that the presence phenanthroline facilitated the splitting off iron from the transferrin. That the trans- rin was saturated with iron also has yed a part.

Rate of binding of iron to transferrin

The first step in the determination of the nsferrin concentration in serum was that saturating all the transferrin with iron

Table II. *The influence on the determination of the total iron-binding capacity of the time of standing after the addition of iron*

Time of standing Min.	Total iron-binding capacity (mean) μg per cent	Serum No.
0	295	1
5	358	1
1	322	2
5	320	2
5	317	3
60	318	3

The determination of the total iron-binding capacity of serum (mean of 4 to 6 determinations) is influenced by the time elapsing between addition of iron and addition of phenanthroline to serum.

by adding a ferro-iron solution to the serum. Next phenanthroline was added to bind the excess of iron. This was not to be done till the transferrin had been saturated, as transferrin is unable to liberate iron already bound to phenanthroline. Laurell did not indicate exactly how long the interval ought to be between the addition of iron solution and that of phenanthroline solution. Table II shows how the values for the total iron-binding capacity were influenced by altering the time elapsing between the addition of iron and that of phenanthroline.

The iron-binding capacity was examined in a serum to which phenanthroline had been added immediately and 5 minutes after the iron. The iron-binding capacity was highest in the latter case. When adding phenanthroline immediately after the iron, the transferrin had not yet been saturated with iron, and some of the iron which by longer standing would have been bound to transferrin was now bound to phenanthroline. The iron-binding capacity was therefore here deter-

Table III. *Influence of the added amount of sodium hydrosulphite*

Added amount of sodium hydrosulphite (mg per ml)	Total iron-binding capacity μg per cent
0	323
1	314
2.5	317
5	302
7.5	238
10	191

The influence of sodium hydrosulphite added in increasing quantities on the determination of total iron-binding capacity.

mined too low. The analysis also showed that iron did not change from phenanthroline-binding to transferrin-binding. The iron-binding capacity in two other sera was not altered by varying the interval between iron and phenanthroline addition from 1 minute to 1 hour. This observation shows that iron bound to transferrin is not liberated by standing and bound to phenanthroline.

The binding of iron to transferrin proved not to occur instantaneously, but still so rapidly that at least 5 minutes' standing after addition of iron sufficed.

3. *Significance of sodium hydrosulphite*

Small quantities of sodium hydrosulphite were added to ensure that the iron was kept in reduced form. Table III shows the effects of varying amounts of hydrosulphite on the iron-binding capacity. Addition of up to 3 times the normal amount of sodium hydrosulphite proved to have no influence, whereas addition of larger quantities gave too low values for the iron-binding capacity, presumably due to lowering of the pH.

Table IV. *Influence of time on iro phenanthroline binding*

Time of standing of the sample after addition of phenanthroline Minutes	Total iron-binding capac	
	Serum I μg per cent	Serum μg per c
0	329	337
10	302	302
30	291	285
60	288	291
120	291	288

The determination of the total iron-binding ca city of serum is influenced by the interval betwe addition of phenanthroline and sodium hydrosulph and addition of hydrochloric acid.

4. *Rate of binding of free iron to phen throline*

Table IV shows how the determinatio influenced by variations of the interval tween addition of phenanthroline and sodi hydrosulphite and addition of hydrochlo acid. It is seen that an interval of 10 minu was too short, whereas 30 minutes suffic and 2 hours gave no further binding.

5. *Stability of the ferrophenanthrolin compound*

We know from ordinary serum iron det minations that addition of hydrochloric a and trichloracetic acid to serum liberates the protein-bound iron. Highly acid re tions, however, also proved to split fer iron from phenanthroline. At room tem rature this splitting was so slight that it of no importance during the experime period. Variations in the added iron amou at least within narrow limits, thus pla no part.

The experiment demonstrating this was as follows (Table V). 2.5 ml of

able V. *Extinction of a solution of rrophenanthroline after addition of hydrochloric acid*

Time after hydrochloric acid addition Minutes	Bound ferro-iron μg per cent
0	228
10	226
20	224
40	219
60	211
80	209

Amount of phenanthroline-bound iron in 5 ml of rophenanthroline solution found after addition of ml of 6-normal hydrochloric acid.

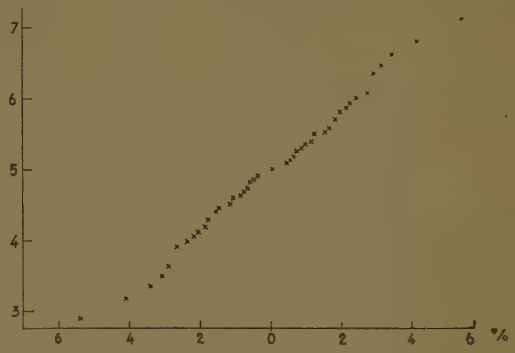


Fig. 2. Probit diagram showing the percentage deviations of duplicate determinations of the total iron-binding capacity of serum. Ordinate: probit value, abscissa: percentage deviation.

rmal hydrochloric acid were added to 5 ml ferrophenanthroline solution, and the extion was now read at regular intervals. his was an expression of the rate of splitg of the ferrophenanthroline compound, e split compound being colorless. The first minutes the splitting was rather inconderable, but still so great that the time nits indicated had to be kept to avoid rors due to splitting of the ferrophenanroline compound. The splitting was, however, less marked in serum, due, among ner things, to the buffer action of serum. The rest of the determination of the ironnding capacity of serum was carried out an ordinary serum iron determination th phenanthroline.

Analysis of solutions with known iron ncentration: Iron concentration 293 μg r cent: Extinction (mean): 0.2518. Consrsion factor: 1163.

The color reaction was in keeping with er's law. Fig. 2 is a probit diagram showg the percentage deviation of 29 random plicate determinations of the iron-binding capacity. The percentage deviation follows the binomial law of distribution. The estimate of the standard error of the individual observations is ± 2.2 per cent, and the standard error of the duplicate determinations $\frac{\pm 2.2}{\sqrt{2}}$ per cent. The percentage deviation does not depend on the total iron-binding capacity.

The total iron-binding capacity and serum iron were determined for 20 normal men and 20 normal women. All the subjects stated that they were feeling perfectly well, more particularly that they were completely free from infections. The Hb per cent and the E.S.R. were examined and found normal. The mean for the women was 309 μg per cent with a standard deviation of the individual observations of ± 27 μg per cent (19 degrees of freedom) and for the men 311 μg per cent with a standard deviation of the individual observation of ± 28 μg per cent (19 degrees of freedom). Thus, the men and the women showed no difference

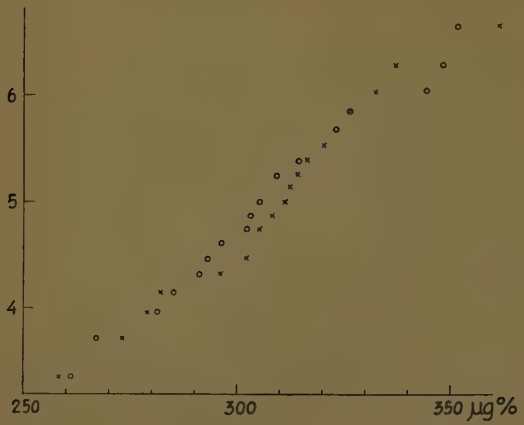


Fig. 3. Probit diagram showing the distribution of determinations of the total iron-binding capacity of serum in normals. Ordinate: probit values, abscissa: total iron-binding capacity in µg per cent (women O, men ×).

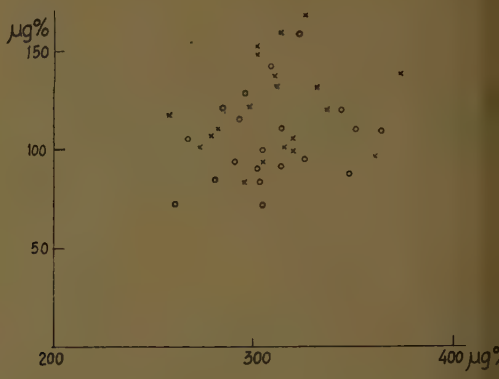


Fig. 4. Point diagram showing determinations serum iron and total iron-binding capacity in no mals. Ordinate: serum iron in µg per cent, abscissa total iron-binding capacity in µg per cent (women O, men ×).

in total iron-binding capacity. The distribution follows the binomial law (Fig. 3).

The total iron-binding capacity of serum determines the upper limit of the serum iron concentration, but seems otherwise, according to Laurell, to have no influence on the serum iron level. This depends on the iron requirement of the organism and the iron reservoirs: If the iron reservoirs are filled to excess, the total iron-binding capacity is saturated while in iron deficiency the percentage saturation is very low. The relative iron saturation of transferrin being thus determined by the iron requirement of the organism, and somehow or other balanced with the reservoirs, the possibility might be conceived that a rise of the serum transferrin concentration causes a rise of the serum iron and *vice versa*.

Fig. 4 shows the relation between the total iron-binding capacity and the serum iron. The variation is considerable, and no definite relation between the total iron-bindi capacity and the serum iron can be see The relative iron saturation of transferr (= serum iron in per cent of the total iro binding capacity) in sera from the prese series of normal men (19 degrees of fre dom) and Laurell's series of normal m (60 degrees of freedom) is 39 per ce while Rath & Finch (1949) find a relati iron saturation in their series of normal m (14 degrees of freedom) of 34 per ce The same investigators and also Ventura Klopper (1951) find the same mean val for percentage iron binding in their seri of normal women, 34 per cent.

SERUM COPPER IN NORMALS

Warburg (1927) was the first to dete mine the serum copper concentration in fairly large number of persons. He e ployed a catalytic method. The most impo tant subsequent determinations have be

blished by Cartwright (1950). The results, least of the subsequent determinations, e fairly alike. The average value seems to slightly higher for women than for men.

Present investigations

For serum copper determination I used vin Nielsen's (1944) method, in a modi-cation making it easier combined with rum iron determination.

METHOD

Reagents:

Hydrochloric acid for analysis, 6-normal.
Trichloracetic acid for analysis, 20 per cent.
Potassium carbonate for analysis, 50 per cent ution, with 4 per cent sodium pyrophosphate ded, for analysis.
Sodium diethyldithiocarbamate for analysis 0.5 r cent solution in redistilled water.
Isoamyl alcohol for analysis.
The chemicals were purified as indicated by vin Nielsen (1944).

PROCEDURE

To 3 ml of serum in a round-bottomed centri-gal test tube I added 1.5 ml of 6-normal hydro-loric acid. The tube was left stoppered with a raffinised cork at 37° C for one hour. After oling, 3 ml of 20 per cent trichloracetic acid was mixed by shaking, the tube being closed by a ger pulp. The precipitate was then carefully tached from the walls of the tube with a glass d. After 10 minutes standing followed centrifuga-n at 3000 rotations per minute for 20 minutes. ml of the centrifugate were mixed with 2 ml 50 per cent potassium carbonate solution, to ich had been added 4 per cent sodium pyrophos-ate. After shaking, 2 ml of isoamyl alcohol d 0.4 ml of 0.5 per cent sodium diethyldithio-rbamate were added, and then followed thorough aking for 1.5 minutes. After the amyl alcohol d separated by standing, the water suspended in was removed by centrifugation for 10 minutes. ien the amyl alcohol was pipetted off and trans-rred to a 2 cm semimicrocuvette, and reading was rformed in a Pulfrich's stufen-photometer with ter S_{43}.

Table VI. *Serum copper determination by Levin Nielsen's method (wet ashing) and own modification (trichloracetic acid precipitation)*

Serum No.	1 μg%	2 μg%	3 μg%	4 μg%
Serum copper (wet ashing)	114	151	157	124
Serum copper (trichloracetic acid precipitation)	113	150	161.5	123

Comparison of serum copper determinations by Levin Nielsen's method (wet ashing) and own modification (trichloracetic acid precipitation).

Analysis of solutions with known copper concentrations. Copper concentration: 100 μg per cent. Extinction: 0.192. Conversion factor: 520.

There was fair accordance with Beer's law.

Added amounts of copper could be found quantitatively.

No difference was observed (Table VI) between the results of serum copper determinations by Levin Nielsen's method (wet ashing) and my own modification (trichloracetic acid precipitation).

Serum copper was determined on sera from the same 40 men and 40 women whose serum iron values were determined. The mean value for the men was 113.0 μg per cent, with a standard deviation of the individual observation of ± 16.4 μg per cent, and for women 121.6 μg per cent, with a standard deviation of ± 16.7 μg per cent. The distribution follows approximately the binomial law. The difference in mean value for men and women is investigated by means of the V^2 method, the probability is between

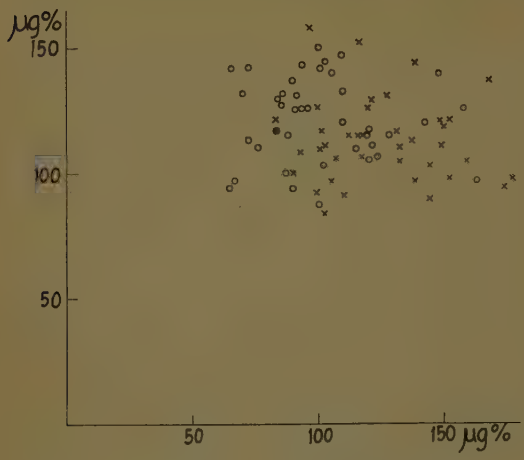


Fig. 5. Point diagram showing determinations of serum copper and serum iron in normals. Ordinate: serum copper in µg per cent, abscissa: serum iron in µg per cent (women O, men ×).

97.5 per cent and 99 per cent. The difference is so small and the variation so great that the question has no clinical importance.

On the basis of duplicate determinations the standard error of the individual observation is calculated at ± 1.7 per cent (and of the duplicate determinations at $\frac{\pm 1.7}{\sqrt{2}}$ per cent) (25 degrees of freedom). Fig. 5 shows no relationship between the serum iron and serum copper concentrations.

SUMMARY

The results are reported of determinations of serum iron, the total iron-binding capacity of serum, and serum copper in normals, and the methods employed are described. The serum iron concentration proved to be highest in males, the total iron-binding capacity alike in males and females. No relationship was observed between the serum iron and serum copper values by simultaneou determination, and no definite relation ca be seen between the values of serum iro and total iron-binding capacity of serum.

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NFLUENCE OF THE SURFACE TENSION IN SERUM AND URINE ON THE FLAME-PHOTOMETRIC ANALYSIS

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(Received for publication Mars 20, 1953)

A number of substances can lower the rface tension of fluids. Among these subances, however, alcohols can give rise to positive error in flame-photometric anases carried out without an internal stanrd (Beckman, 1947; Berry, Chappell & arnes, 1946 and Gilbert, Hawes & Beckan, 1950), whereas certain organic acids d other substances do not interfere with e accuracy of the analytical results — ssibly on account of a time factor (Hasler, 40 and Berry, Chappell & Barnes, 1946). The surface tension of serum and urine n vary considerably (Adlersberg & Singer, 25, Earlam & Morton, 1935, Perryman & lons, 1935 and Posner, 1951). Surface nsions as low as 47 dyn/cm have been easured on normal serum, 50 dyn/cm on rmal urine. Serum from a nephritic patient s shown a surface tension as low as 44 n/cm, and the urine 46 dyn/cm (Matuda, 38). Even when the urine is diluted 1 :100, e surface tension is still lowered (Leiter, 26).

In order to see whether the capillary-tive substances occuring in serum have to classified in this respect with alcohols or th the other group, the following experients were made.

TECHNIQUE

The sodium analyses were performed by means of the flame photometer described previously, with an average standard deviation of ± 0.9 per cent (Ryssing, 1952). The apparatus can be adjusted to work with and without an internal standard. The surface tensions were measured with a modified DuNoüy apparatus about 30 sec. after the formation of the surface, and converted to dyn/cm.

EXPERIMENTS

Seven solutions were prepared with 0—2.5—5.0—7.5—10.0—12.5—15.0 per cent propyl alcohol, all of them containing 1.5 mg per cent sodium chloride and 100 mg per cent lithium chloride. The surface tensions were measured and the solutions analyzed in the flame photometer with and without the corrective influence of an internal standard. Fig. 1 shows that the results are independent of the surface tension when the analyses are carried out with internal standard, but encumbered with an increasing positive error with falling surface tension when the analyses are carried out without internal standard.

Then 4 icteric sera were examined, diluted 1 : 200, and lithium chloride was added at a

The work here reported was carried out with the aid of a grant from P. Carl Petersens Fond.

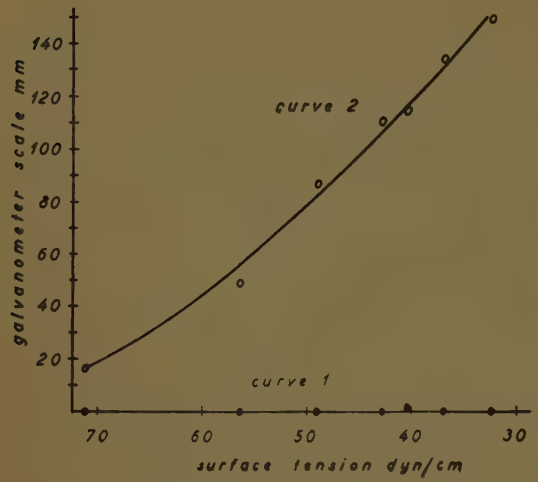

Fig. 1. The influence of the surface tension upon the galvanometer reading. The surface tension is lowered by adding increasing amounts of propyl alcohol to 6 solutions with the same sodium concentration. Abscissa: surface tension in dyn/cm. Ordinate: galvanometer reading in mm. 10 mm = 0.96 meq/l sodium in serum at a dilution of 1:200. While the surface tension does not induce any error when the analyses are carried out with the correcting influence of the lithium light (curve 1) an increasing error is found with declining surface tension (curve 2) working without internal standard.

concentration of 100 mg per cent. Table 1 shows that the surface tension of the serum dilutions is lower than that of the standard solution, and that the measured sodium concentrations are independent of whether the analyses were carried out with or without the influence of internal standard.

According to curve 2 in Fig. 1, it was to be expected that the sodium concentration measured in serum No. 1, in the analysis without internal standard, would be 2.7 meq/l higher than in analyses without internal standard.

Table I. *Table I shows the results of th low surface tensions of the serum dilution not influencing the analyses if these ar carried out without the correcting influenc of the lithium light. The four icteric ser are diluted 1: 200*

	Surface tension dyn/cm	The analyses are carried out: with internal standard meq/l	The analyses are carried out: without internal standard meq/l
Standard solution ..	71.5		
Serum No. 1	57.8	140.0	135.2
— 2	62.0	143.4	143.6
— 3	65.2	138.7	137.4
— 4	60.7	142.1	142.6

DISCUSSION

Undoubtedly the explanation of the findings is to be looked for in the fact th the surface tension of certain solutions *e. g.*, serum and urine dilutions — is d pendent upon the length of time for whi the surface has persisted, in contrast to t surface tension of solutions containing alc hol, phenol or glucose (Perryman & Selor 1935, and Du Noüy, 1929). Owing to t high rate of outflow of the fluid through t efflux opening, the capillary-active su stances presumably have not time enough exert their influence.

SUMMARY

The experiments show that the low su face tension that may occur in diluted icte sera (probably also in specimens of urin have no effect on the flame photomet analysis, whether or not this is carried with internal standard. As pointed out fore (Ryssing, 1952), for other reasons is of advantage to use internal standard

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LONG TERM ANTICOAGULANT TREATMENT OF CARDIOVASCULAR DISORDERS VITH PHENYLINDANDIONE (PID-TROMBANTIN)

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(Received for publication June 6, 1953)

Anticoagulent treatment in cardiovascular disorders has chiefly been confined to acute intravasal occlusion, particularly in the coronaries and the peripheral muscular arteries. Owren and Aas, however, have shown that this form for therapy of coronary disease can be carried on for months, even years, without hemorrhagic complications or secondary thromboembolic episodes occurring (Owren & Aas, 1951). The technic is dependant on reliable laboratory measurements and full knowledge of the pharmacological characteristics of the drug (Blumgart, 1952). Bjerkelund has recently discussed this form of treatment and its immediate results for a shorter period of observations (Bjerkelund, 1953).

The purpose of this paper is not to bring the justification for this form of treatment into discussion. Although promising, it is still *sub judice*. My purpose is to call attention to the method of administration of an anticoagulant for use in ambulatory practice. The drug used is phenylindandione, (PID), brought on the market under the name Trombantin. Owren (1952) has shown that the response to PID is similar to that to dicumarol, *viz.:* it will reduce the proconvertin as well as the prothrombin concentration. The action of this drug is quicker than that of dicumarol, giving maximal r duction of the prothrombin values about 4 hours after the administration. Bjerkelun (1953) has found the immediate response t single doses of PID to be quite stabl whereas the responses to single doses c dicumarol were widely divergent.

LABORATORY METHODS

The control of proconvertin and prothromb values (P & P-values, Owren & Aas, 1951) h been carried out in the laboratory of P. A. Owre The curves give a convincing illustration of t exactness of this method.

DOSAGE

After a trial period the following schedu was adopted: First day 150 mg (3 tablets followed by a dose of 100 mg each mornin Third day control of P & P-values. Fig. shows the uniform response to this dosag No correlation has been found between t initial P & P-values and the concentrati registered the third day of treatment (Fig.2 (It will be noted from the curves that t more the P & P-values are reduced, the le will the same dose of PID reduce the P & concentration, a well known experience fro other antienzyme activity.) Except for t two first cases, all the patients have be

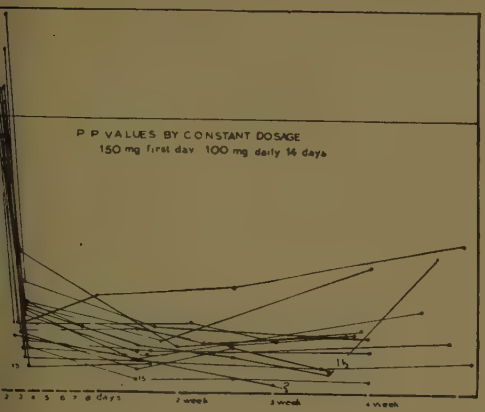

Fig. 1.

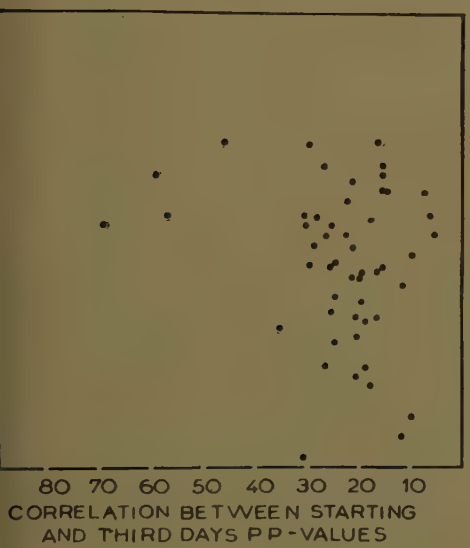

Fig. 2.

ated ambulatory from the first day.[1] The ction to treatment has been suprisingly stant: of 55 patients treated over a period six months or more, 37 have been kept on

[1] Hospitalized patients show lower tolerance for drug. The average starting dose for these ents probably lies about 100 mg (E. Vogt, per- al communication).

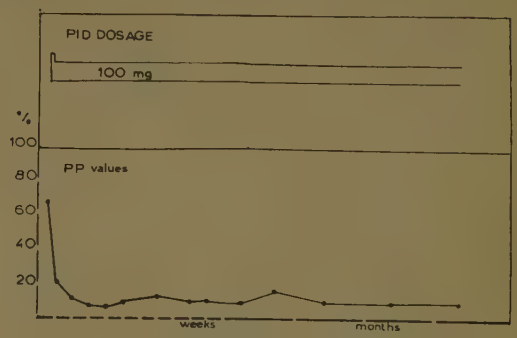

Fig. 3 a.

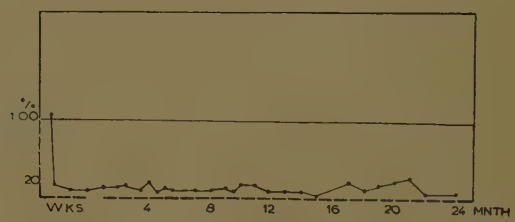

Fig. 3 b.

constant dosage for two months or more, with very small variations of P & P values, most of which were kept constant between the optimal ranges (10 to 30%). Fig.3a and b. In 6 patients constant dosage has been kept for twelve months or more, the average duration of constant dose for this group of patients being 7 months. As for dicumarol, the dose of PID necessary for keeping the patients within optimal P & P values varies from patient to patient within a range from 50 up to 225 mg a day. It has been the rule to give the patient the whole dose early in the morning.

Some patients will show an increasing tolerance to the drug, necessitating augmentation of dosage (Figs. 4 & 5). This increased tolerance can be lost after two months pause in treatment (Fig. 6). Of the

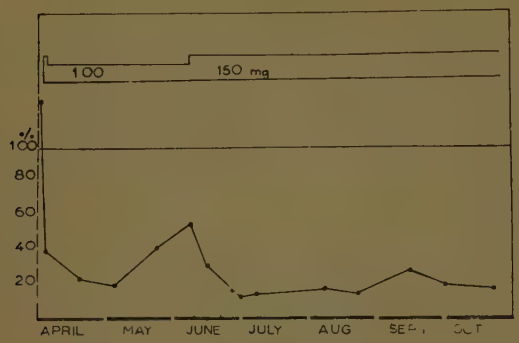

Fig. 4.

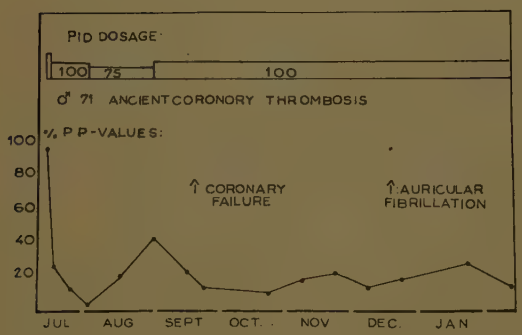

Fig. 5.

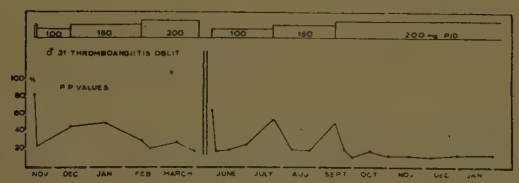

Fig. 6.

55 patients, 15 showed this increased tolerance during treatment. This tendency can be found early in treatment, or after several months (up to 9 months) of constant dosage on a lower level. Only 4 patients showed tendency to reduced tolerance, inducing reduction of dosage. The reduction was always very slight, (25—50 mg) and was never forced by bleeding tendency.[2]

COMPLICATIONS

Among our first cases, three complications of more severe nature were encountered. One patient developed severe intraperitoneal bleeding and was operated upon under the diagnosis acute appendicitis. This patient has been kept on constant PID dosage for 6 months after the acident without further complications. Two patients developed gross hematura. The first patient was switched over from dicumarol, the effect of the more long-acting drug being added to that of PID confused the habitual response. Apart from these early accidents, the complications have been negligible (slight nose bleeding, slight tendency for bleeding while shaving). One patient developed a stomatitis but it has not been established that this was due to the drug. One patient with angina pectoris died after two months of treatment. His PP values were increasing, the last check up showed 37 %. He was planning a 4 weeks holiday in the mountains, and the dosage of PID, therefore, was not augmented. He dropped dead one week after the last control. Three patients got symptoms indicating coronary failure during treatment. One patient died from hemorrhage from malignant brain tumor.

PHARMACODYNAMIC EFFECT

It has long been known that some of the patients suffering from coronary disease develop greater tolerance to work during

[2] Caution: A drop in P & P values can follow treatment with antibiotics such as aureomycin (Owren, personal communication).

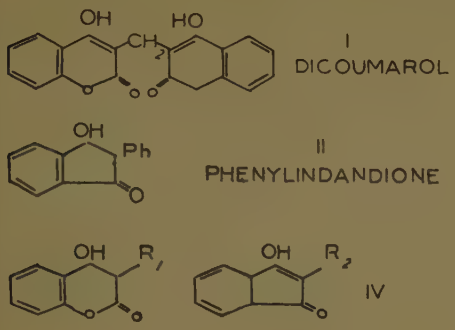

Fig. 7.

reatment with dicumarol (Gilbert, 1952). his effect can be found even with PID. onsidering the intimate constitutional rela- onship between the two drugs this is not stonishing (Fig. 7). Of 33 patients suffering rom coronary disease the anginal pain was iminished in 26, with increased tolerance to xercise. Only 7 were unchanged. Of 22 atients with obliterating arterial disease 21 ere symptomatically improved, some became mptom-free. This symptomatic relief could e demonstrated very early in some cases: articularly in patients treated for imminate angrene or chronic torpid ulcers of fingers r toes. In the majority of patients the in- reased tolerance to exercise was established fter several weeks or several months.

The similar responses to PID and to cumarol will, however, not justify the nclusion that the effect of one dose of e former corresponds to the effect of similar dose of the latter. I had had vo patients switched over from dicumarol PID, one of whom had been treated for months with doses about 62.5 mg of dicumarol, the other being treated with only 37.5 mg, the two of them being stabilized on *the same* dose of PID: 75 mg.

COMMENT

Arteriosclerosis represents the "big killer" of men of the western hemisphere. No clue has so far been found for the solution of the pathogenesis of the disease, and no prophylaxis can be described. But one thing has been established: The alarming complications and fatal outcome are frequently caused by thrombosis. This point must be stressed without bringing Duguids (1952) interesting theory into discussion. It is also seems very likely that intra-arterial clot formation plays an important part in anginal pain and intermittent claudication (Pickering, 1951).

The long-term anticoagulant treatment of cardio-vascular disorders represents a valuable aid in our effort to fight the thrombolic complications of arterioscloresis. It is, of course not *the solution* of the whole problem.

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β-GLOBULIN CONCENTRATION AND TREATMENT OF INFANTILE LEUKEMIA

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(Received for publication July 11, 1953)

The methods for the characterization of such classes of substances as proteins and lipids have in later years been greatly improved. Above all, better methods for micro-analyses have been elaborated, a most important factor in pediatrics and in many cases an absolute prerequisite for research in this field.

For 18 months, nine cases of infant leukemia have been closely followed. This paper mainly gives observations on the protein fractions in the blood of these children, as obtained by micro-electrophoresis according to Antweiler. In connection with these investigations some lipid-analyses also touched upon have been performed.

1. *Protein analyses*

Great changes often occur in the serum protein of leukemia patients. Most obvious is a decrease in the albumin/globulin ratio and alterations of the amounts of the globulin fractions. The normal values for the globulin fractions in a veronal buffer of pH 8.6 are according to Dole (1944): α and β each about 12 per cent, γ about 11 per cent. The few publications available, containing results from electrophoretic investigations in cases of child leukemia, indicate a variation mainly in the γ-globulin (Brown Read, Wiseman & France, 1948).

Eight of the nine cases studied her showed a content of α-globulin of above 2 per cent, three as high as 40 per cent. Suc an increase in the concentration of α-globu lin is often found in connection with othe system diseases. As in earlier published in vestigations, variations in the γ-content hav also been observed, both abnormally hig values of up to 40 per cent and abnormall low values down to 4 per cent. The behavio of the β-fraction during the course of th disease seems more significant. Seven of th nine cases studied showed an abnormal low β at the beginning of the observatio period: five cases below 2 per cent, tw cases below 4 per cent. The two others ha a normal β, i. e. about 12 per cent. The tot protein content of the serum has general been normal.

An obvious thing to do was, of course, find out what would happen on the additio of β-globulin, e. g. by a blood-transfusio Strangely enough it was found that in tw cases particularly studied in this connectio the β-globulin percentage remained low ev after repeated transfusions. It seemed as the β-globulin supplied was immediate consumed and it was therefore thoug

sirable to try β-globulin in larger doses. B Kabi supplied a fraction consisting inly of the metal-binding β-globulin ich represents 25 per cent of the β- globu- complex. This fraction was prepared cording to the method of Cohn (Cohn, rong, Hughes, Mulford, Ashworth, Melin Taylor, 1946; Cohn, Gurd, Surgenor, Bar- s, Brown, Derouaux, Gillespie, Kahnt, ver, Liu, Mittelman, Mouton, Schmid & roma, 1950) which comprises a fractional ecipitation with alcohol at a low tempera- re, this fraction being designated by Cohn fraction IV—7.

Treatment with metal-binding β-globulin and with zinc

The metal-binding β-globulin has been ven intramuscularly to five of the seven ildren with low β. The other two cases d gone ad mortem before the beginning these experiments. The dosage has been —30 mg/kg body weight/day. It was und that after a 14 days treatment the globulin content of the blood reached an most normal value (Fig. 1). After 1—2 nths of treatment it was possible to halve e dose without any observable permanent crease in the β-globulin. Further, it seems if the α- and γ-globulins were also nor- alized by this treatment. It should also be inted out that plasma cells appear in the ood when β begins to increase or just ter β has reached its constant value. These lls may therefore be regarded as an ndicator" showing that something has ppened. Perhaps the occurrence of plasma lls should be connected with their possible nction as protein producers (Bing & Plum, 1937; Bing, Fagraeus & Thorell, 1945; Fagraeus, 1948).

It should be noted that the indicated dosage implies a daily supply of metal-binding β-globulin corresponding to ⅛—¼ of the amount normally present in the blood of a healthy child. Such a dose cannot of course be administered by normal blood transfusions.

This globulin-fraction thus seems to stimulate a synthesis of the entire β-complex, because in two cases where macro-electrophoresis could be performed it was possible to state that all β-fractions, not only the added one, had increased.

Fig. 1 shows the variations in the β-globulin with time for three cases, immediately before and after the treatment, the beginning of which is marked as day 0. The low β-level before the treatment and, in cases III and V, the considerably higher level stabilized after 14 days are clearly demonstrated. Case IV went ad mortem 1 week after the beginning of the treatment but even here a certain increase in the β-content was observable. Cases III and V received blood transfusions both before the treatment and after β had reached its higher level. During the period of increase no blood was administered. No momentary increase of β was observed in connection with the transfusions. (Case III, showing a Hb of 33 per cent — 5.0 g per cent, received before the treatment in all 50 ml blood/kg body weight. Case V had Hb 12 per cent — 1.8 g per cent and received before the treatment 70 ml blood/kg body weight.)

The vertical staples denote the plasma cells, the height being proportional to the number and each unit designing 1 plasma

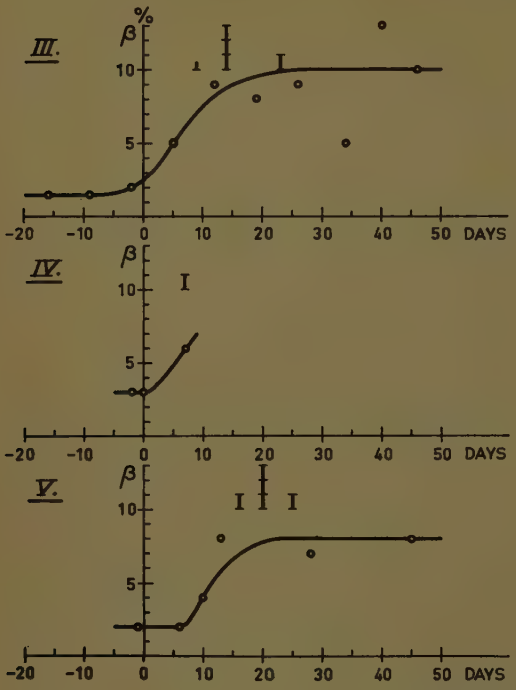

Fig. 1. Concentration of β-globulin before and during the treatment.

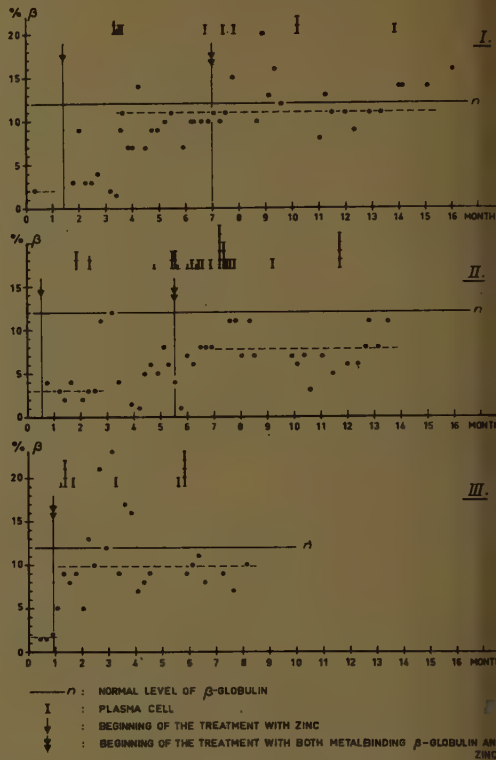
I.
II.
III.
% β
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 MONTH
n : NORMAL LEVEL OF β-GLOBULIN
I : PLASMA CELL
: BEGINNING OF THE TREATMENT WITH ZINC
: BEGINNING OF THE TREATMENT WITH BOTH METALBINDING β-GLOBULIN AN ZINC

Fig. 2. Concentration of β-globulin during a longer period.

cell per 100 white blood cells. The highest staple thus means 3 plasma cells and it is obvious that these staples concentrate around the time of β-increase. Before the treatment no plasma cells had been observed in any of the three cases.

It must be pointed out that cases III and V had received zinc simultaneously with the β-globulin. The reason was that, before treatment with the metal-binding β-globulin became of interest, two earlier cases had been treated with intramuscular injections of 0.1—0.2 mg zinc citrate/kg body weight/day (Fig. 2). As the zinc treatment seemed possibly to have caused a minor increase in β, it was thought that it could not be di carded until further experience had be gained. It is known from earlier investig tions (Vallee & Altschule, 1949; Vallee Gibson, 1948, 1949; Vikbladh, 1951; Be fenstam, 1952) that the plasma zinc is l in leukemia, this being one of the reaso for its administration.

Fig. 2 shows the variation in β-globul over a longer period of time (3 cases). T two levels are clearly visible: a low o occurring before that marked by the dra line. Cases I and II had already been treat with zinc, the start of this treatment be

rked by the single arrow. About six nths later, as marked by the double arrow, tal-binding β-globulin was also admi- tered. The zinc treatment seemed to in- ence the β-fraction somewhat but a stabi- d higher level was not obtained until tal-binding β was given. Case III received h zinc and metal-binding β from the be- ning and the constant β-level was reached ch more rapidly (in about 14 days). The sma cells have been marked on this figure o. In case I and III they occur at the step- se increase of β. In case II they appear re irregularly. The period of observation s 8—16 months. Case I received no blood nsfusion whilst case II received the first nsfusion 7 months after the start of the atment.

The two cases showing normal β in the ginning of the observation were also ated with metal-binding β-globulin and ic during a period of two months. The content remained normal, no change being served.

The β-fraction in relation to the blood lipids

Since it is known that the β-globulin acts a carrier not only for metals but also for e lipids of the blood, it seemed advisable o to pay attention to the lipid content of e blood (cf. Lipo-proteins 1949). The β- bulin, or more particularly the β_1-frac- n, normally contains ¾ of the plasma ids. The remaining quarter is bound ainly to the α_1-globulin (Gurd, Oncley, lsall, 1949.)

In several of the cases referred to above, certain lipemia was observed even after e time when a normal alimentary lipemia should have disappeared. Particularly in two cases a definite lipemic condition was observed where the β-globulin content was low. Total lipids of 1350 and 1950 mg per cent have been determined.

Possibly this demonstrates a disturbance in the normal interchange between the blood lipids and the fat deposits, caused by the low β-globulin content. Thus a diminished interchange of lipids between the blood and other tissues may be due to an increase in size of the lipid particles, probably caused by the low β-content. This particle growth may perhaps become irreversible and subsequently critical.

Case IV, which went ad mortem one week after the start of the treatment, died in symptoms similar to lung embolism, the post mortem, macroscopic examination giving no explanation. Histological examination of lung tissue (fat staining) showed, however, that the capillaries in the walls of the alveoli had largely been filled by fat and thus stood out intensely red (here black) on Sudan-staining (Fig. 3).

4. *Clinical notes*

The children studied here have been diagnosed as acute leukemia of immature character, partly of the reticuloendotheliosis type, i. e. the puncture of the bone marrow showed cells which were less differentiated than the blast cells. Most cases have been of a leukopenic type. During the course of the treatment partial clinical remissions have occurred with simultaneous tendency to normalization of the number of blood cells and maturation of the peripheral blood. Occasionally an increased differentiation has been observed in the sternal punctures.

Fig. 3. Lung tissue with fat embolism.

Since, however, spontaneous remissions occur in leukemia, the material must be regarded as too limited to allow of a judgment concerning the clinical value of treatment.

SUMMARY

Analyses of blood from children with acute leukemia have given the following results:

1) Of the nine cases studied, seven showed an abnormally low β-globulin content: five cases below 2 per cent, two cases between 2—4 per cent and the two others a normal β. Variations have also occurred in the α- and γ-fractions.

2) Five of the cases with a low β received intramuscular injections of metal-binding β-globulin together with zinc (metal-binding β-globulin 20—30 mg/kg bod weight/day, zinc 0.1—0.2 mg/kg bod weight/day). A normalization of the globulin content of the blood followed th treatment and the other globulin fractio also seemed to tend to a normalization. O patient was only given metal-binding This case also showed an increase in the content of the blood.

3) Zinc by itself also seems to influen the protein fractions, though less pronounce than when combined with the metal-bindi β-globulin.

4) Clincal improvement seems to follo the increase of the β-globulin.

5) Plasma cells appear in the blood wh the β-content increases, or just after it h reached a higher level. The appearance plasma cells may perhaps be connected wi their possible function as protein produce

6) A serious lipemia with lethal effe has been observed in one case where the globulin content was low. A freeze-prepar tion of lung tissue, with fat staining, show that the capillaries of the lung alveoli h been largely filled with fat. Possibly this w caused by a disturbance of the normal int change between blood lipids and fat depos caused by the low β-globulin content.

ACKNOWLEDGEMENT

The author wishes to express her sincere gra tude to Professor Curt Gyllenswärd, M.D., for never-failing interest and for stimulating disc sions during the course of her work.

The fractionations have been carried out at A Kabi, Stockholm, and some of the analyses Forskningslaboratoriet LKB, Äppelviken.

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ON THE DETERMINATION OF GLUTAMINE AND GLUTAMIC ACID IN URINE BY A MODIFICATION OF KREBS' METHOD

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(Received for publication July 23, 1953)

A specific method for the quantitative determination of glutamine and glutamic acid has been worked out by Krebs (1948). This method is based on the following principles:

The sum of glutamine and glutamic acid is determined by measuring the amount of carbon dioxide evolved under the influence of Clostridium Welchii SR 12. Glutamine is determined as the ammonia produced in this process. Glutamic acid is calculated as the difference.

In this way the content of glutamine and glutamic acid in several tissues has been determined. (Krebs, Eggleston & Hems 1949) Munkvad (1951) has shown by applying this method, that the concentration of glutamic acid in blood plasma is lower in patients suffering from schizophrenia than in normal control subjects.

These results encourage to further investigations of the intermediary metabolism of glutamine and glutamic acid. The original method of Krebs, however, cannot be used to determine the amount of glutamine and glutamic acid excreted in urine. In the Krebs' method glutamine is determined as the ammonia produced from glutamine under the influence of the glutaminase present in Clostridium Welchii SR 12. Preformed ammonia present therefore always interfere with this determination. In urine the co centration of preformed ammonia ordina ranges between 20 and 100 mg pr. 100 m while the concentration of the ammon evolved from the glutamine present urine never exceeds 2 mg pr. 100 ml urin (According to the authors investigations It is impossible exactly to determine th small amount of "glutamine—ammonia" the presence of the much larger amount preformed ammonia. Therefore, determin tion of glutamine in urine cannot be carri out after the original method of Krebs wit out first removing the preformed ammoni This would, however, complicate the dete mination.

In the following a description is given a modification of the Krebs' method, whi makes it possible to determine both glutami and glutamic acid independent of preform ammonia present. This new method is bas on the following principles:

Glutamine, but not glutamic acid, is co verted to pyrrolidonecarboxylic acid means of heating to 100° C under speci circumstances. As the method of Krebs highly specific for glutamine and glutam acid, pyrrolidonecarboxylic acid is not dete mined in this method. Therefore, when

ution containing both glutamine and glut-
ic acid, undergoes this process of heat-
, glutamic acid remains as the only sub-
nce which is determined by the Krebs'
thod. Thus it is possible to determine
tamic acid independently of glutamine and
formed ammonia present.

Further on: The sum of glutamine and
tamic acid in urine is determined without
ficulty according to Krebs. Glutamine,
n, is calculated as a difference.

The principles of this new method shortly
:

In one fraction of urine the sum of gluta-
ne and glutamic acid is determined accord-
to Krebs: Manometric determination of
carbon dioxide evolved at pH 4.9 under
influence of the enzymes present in
stridium Welchii SR 12.

In another fraction the content of glutamic
d is determined in the same way after
ing converted the glutamine present to
rrolidonecarboxylic acid.

The glutamine is calculated as the dif-
ence.

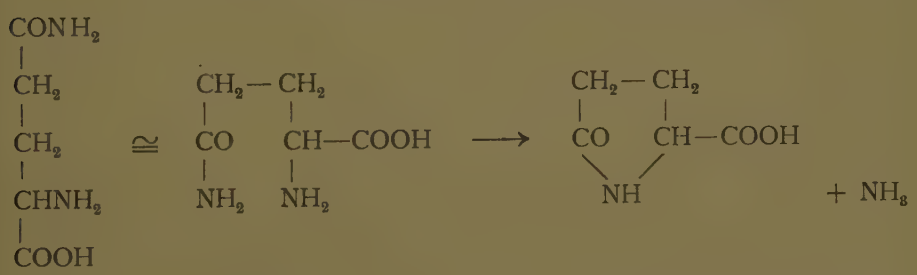
In this way the determination of both
tamine and glutamic acid in urine can be
ried out independently of preformed am-
nia present.

Glutamine, in solution, is converted to pyrrolidonecarboxylic acid by heating to 100° C. The presence of phosphate highly catalyzes the rate of conversion. The pH optimum of this process is about 6.5. In the presence of 0.2 M phosphate buffer pH 6.5 glutamine is completely converted to pyrrolidonecarboxylic acid after being incubated at 100° C for about 40 minutes. (Waelsch 1952). Glutamic acid practically remains unchanged by this process. (Waelsch 1952). Pyrrolidonecarboxylic acid does not evolve carbon dioxide under the influence of the enzymes present in Clostridium Welchii SR 12, as glutamine and glutamic acid do.

The author has in this connection done the following investigations:

Several test tubes containing 0.5 mg glutamine dissolved in 2 ml of 0.2 M phosphate buffer pH 6.5 were incubated at 100° C for different periods of time. After cooling and reducing the pH to 4.9 the content of each test tube was transferred to the main chamber of a Warburg vessel. The side chamber contained 0.5 ml of a 2 per cent suspension of Clostridium Welchii SR 12. Three vessels were used in each deter-

The conversion of glutamine to pyrrolidonecarboxylic acid



Glutamine Glutamine Pyrrolidonecarboxylic acid

mination, all containing the same fluids. When thermal equilibrium was obtained, 40° C, the bacterial suspension was added to the content in the main chamber of two Warburg vessels. The third vessel, in which the content remained unmixed, served a combined blank and thermobarometer. Th amount of carbon dioxide produced wa measured, and the following results we obtained:

Table I. *Quantitative determination of 0.50 mg glutamine by the method of Krebs. The glutamine dissolved in 0.2 M phosphate buffer pH 6.5. The effect of incubating this solution of glutamine at 100° C previous to the determination*

Incubated at 100° C for	0		10		20		40 minutes	
Analysis no.	1	2	3	4	5	6	7	8
Carbon dioxide evolved (mm^3) ..	76	77	33	34	16	15	0	0
Equivalent with mg. glutamine ..	0.496	0.502	0.215	0.222	0.104	0.098	0	0
This corresponds in per cent of the original amount of glutamine	99.2	100.4	43.0	44.4	20.6	19.6	0	0

Conclusion: Glutamine dissolved in 0.2 M phosphate buffer pH 6.5 is, after being incubated a 100° C for 40 minutes completely indifferent in, and is not determined by the method of Krebs.

The resistance of glutamic acid towards being incubated at 100° C at pH 6.5 is shown in the following:

A series of investigations just as the ones described above were carried out with glu amic acid instead of glutamine. Each te tube contained 0.84 mg glutamic acid. Th results were:

Table II. *Quantitative determination of 0.84 mg glutamic acid by the method o Krebs. The glutamic acid dissolved in 0.2 M phosphate buffer pH 6.5. The effect of incubating this solution of glutamic acid at 100° C previous to the determinaton*

Incubated at 100° C for	0			60 minutes		
Analysis no.	1	2	3	4	5	6
Carbon dioxide evolved (mm^3) ...	128	128	129	128	128	127
Equivalent with mg. glutamic acid	0.84	0.84	0.846	0.84	0.84	0.834
This corresponds in per cent of the original amount of glutamic acid	100	100	100.7	100	100	99.3

Conclusion: Glutamic acid remains unchanged in the method used to convert glutamine to py rolidonecarboxylic acid.

Attemps to determine glutamic acid in a ution of pure amino acids, among them tamine.

A phosphate buffer 0.2 M pH 6.5 was pared, each ml containing:

Glutamic acid	0.194 mg
Glutamine	0.88 mg
Arginine	1.02 mg
Glycine	9.50 mg

Test tubes containing 2.00 ml of this ution were incubated at 100° C for one ur. After cooling and reducing the pH to the content of each test tube was trans- rred to the main chamber of a Warburg ssel. The side chamber contained 0.5 ml a 2 per cent suspension of Clostridium

able III. *Quantitative determination by Krebs' method of 0.388 mg glutamic d dissolved in a solution of pure amino d among them glutamine, buffered with osphate 0.2 M pH 6.5. The solution in- bated at 100° C for one hour previous to the determination*

Analysis no.	1	2	3	4
bon dioxide evolved (mm^3)	58	58	59	58
iivalent with mg. glutamic cid	0.380	0.380	0.387	0.380
s corresponds in per cent f the original amount of lutamic acid	97.9	97.9	99 7	97.9

Conclusion: The carbon dioxide evolved prac- lly corresponds to the original amount of glut- ic acid. Thus it is possible to determine glutamic l directly and independently of the glutamine sent by the above described modification of ebs' method.

Welchii SR 12. When thermal equilibrium was obtained, the bacterial suspension was added to the solution of the amino acid in the main chamber. Parallels in which the content in the Warburg vessel remained unmixed, served as combined blanks and thermobarometers. The amount of carbon dioxide produced was measured and the results given in Table III were obtained.

Quantitative determination of glutamic acid in urine

The determination is carried out thus:

KH_2PO_4	0.35 g
Na_2HPO_4 2 H_2O	0.20 g
Urine	20 ml

Shake until dissolved. (The molar concentration of phosphate herby increases to 0.2 and the pH is established at about 6.5.)

Incubate at 100° C for one hour. After cooling the pH is reduced to about 4.5 by adding 10 per cent hydrochlorid acid (0.7 to 1.2 ml) 0.5 ml 3 M acetatebuffer pH 4.9 and 0.05 ml 20 per cent "Cetavlon" are then added. ("Cetavlon" (Imperial Chemical Pharmaceuticals Ltd.) serves as accellerator for the decarboxylation of glutamic acid. (Krebs 1948)). Distilled water is filled up to 25 ml. The solution is mixed and shaken in vacuo in order to remove carbon dioxide.

4 ml of this solution, which contains 3.2 ml urine, is transferred to the main chamber of Warburg vessel. The side chamber contains 0.5 ml of a 3 per cent suspension of Clostridium Welchii SR 12. Three vessels are used, all containing the same fluids. After thermal equilibrium is obtained, 40° C,

the bacterial suspension is added to the urine in two of the Warburg vessels. The amount of carbon dioxide produced is measured. The third vessel in which the content remains unmixed serves as combined blank and thermobarometer.

In order to control this method determin tions were carried out both in urine, a parallel to this, in the same urine to whi known amounts of glutamine and glutam acid were added.

Table IV. *Quantitative determination of glutamic acid in urine by the method of Krebs after having converted the glutamine present to pyrrolidonecarboxylic acid. Parallels in which known amounts of glutamine and glutamic acid were added to the urine.*

Analysis no.	1		2		3		4		5		6		7		8	
Urine (ml)	20		20		20		20		20		20		0		0	
Glutamic acid added (mg)	0		0		0.84		0.84		1.68		1.68		0.84		0.84	
Glutamine added (mg)	0		0		1.90		1.90		3.80		3.80		1.90		1.90	
Distilled water (ml)	0		0		0		0		0		0		20		20	
Carbon dioxide (mm^3) evolved from 3.2 ml urine	1.5	1.5	1.5	1.5	20	20	21	21	39.5	40	41	40	20	20	20.5	
Corresponding amount carbon dioxide evolved from 20 ml urine	9.4	9.4	9.4	9.4	125	125	131	131	247	250	256	250	125	125	128	
Equivalent with mg glutamic acid	0.06	0.06	0.06	0.06	0.82	0.82	0.86	0.86	1.62	1.64	1.68	1.64	0.82	0.82	0.84	
Amount of glutamic acid added to the urine recovered (mg)					0.82 −0.06 0.76		0.86 −0.06 0.80		1.63 −0.06 1.57		1.66 −0.06 1.60					

The added amounts of glutamic acid were to all practical purposes recovered. The glutamine adde did not influence the determination of glutamic acid. Thus determinations of glutamic acid i urine can be carried out by the above described modification of Krebs' method.

Quantitative determination of the sum of glutamine and glutamic acid in urine

The determination is carried out in this way:

Urine	20 ml
Reducing pH to about 4.5 by adding 10 per cent hydrochlorid acid	0.1 to 0.5 ml
Acetate buffer 3 M pH 4.9	0.5 ml
"Cetavlon" 20 per cent	0.05 ml

Distilled water is filled up to 25 ml. T solution is mixed and shaken in vacuo order to remove carbon dioxide.

4 ml of this solution, which contains ml urine, is transferred to the main chamb of Warburg vessel. The side chamber co taining 0.5 ml of a 3 per cent suspension Clostridium Welchii SR 12. Three vesse containing the same fluids, are used. T amount of carbon dioxide evolved in t

rburg vessels is measured. The third ves- serves as combined blank and thermo- ometer.

n order to control the method determina- tions were carried out both in urine and, parallel to this, in the same urine to which known amounts of glutamine and glutamic acid were added.

able V. *Quantitative determination of the sum of glutamine and glutamic acid in ine by the method of Krebs. Parallels in which known amounts of glutamine and glutamic acid were added to the urine*

Analysis no.	1		2		3	
Urine (ml)	20		20		0	
Glutamic acid (mg)	0		0.42		0.42	
Glutamine (mg)	0		0.95		0.95	
Distilled water (ml)	0		0		20	
Carbon dioxide (mm^3) evolved from 3,2 ml urine	38	38	72	71	34	33
Corresponding amount carbon dioxide evolved from 20 ml urine	237	237	450	444	212	206
Equivalent with mg glutamine	1.55	1.55	2.94	2.90	1.38	1.34
Amount of glutamine and glutamic added to the urine recovered. (Calculated as glutamine (mg).			2.94 − 1.55 = 1.39	2.90 − 1.55 = 1.35		

The amounts of glutamine and glutamic acid added were recovered. The method of Krebs is well apted to the determination of the sum of glutamine and glutamic acid in urine.

excretion of glutamine and glutamic acid in urine

s the first step in the investigations of amine and glutamic acid excreted in e the daily excretion of these two sub- ces was followed up in two normal males ng a period of about two weeks. Parallel his the pH of the urine was measured the excretion of total-nitrogen and am- ia-nitrogen determined.

is known that glutamine is the main source of the ammonia synthetized in the kidney and excreted in urine (Peters & van Slyke 1946). Therefore, perhaps, varying excretion of ammonia would cause varia- tions in the excretion of glutamine and glut- amic acid. In order to investigate this pos- sibility the amounts of glutamine, glutamic acid and ammonia excreted were compared and, for some days, the production and ex- cretion of ammonia artificially regulated by acidifying and alkalinizing the organism.

Table VI a and VI b. *The daily urinary excretion of glutamine and glutamic acid in two normal males. Compared with the diuresis, the pH of the urine and the amounts of Total-nitrogen and ammonia-nitrogen excreted. See also the figures 1 and 2*

Table VI a

O. P. F. 36 years, male.

Date	Diuresis	pH	Total-nitrogen	Ammonia-nitrogen	Glutamine	Glutamic acid	Remarks
1953 16/6	750 ml	5.5	9.82 g	0.502 g	76 mg	1 mg	
17/6	1400	5.6	11.61	0.464	106	3	
18/6	1040	5.4	10.10	0.558	92	3	
19/6	920	5.5	10.78	0.540	78	3	
22/6	920	5.4	10.02	0.555	79	2	
23/6	2630	6,0	13.10	0.494	97	3	Increasing the diuresis by drinki 2 l water.
24/6	930	6.2	8.45	0.290	79	2	
25/6	1120	7.1	10.30	0.125	81	2	Peroral intake of 10 g $NaHCO_3$ dissolved in 500 ml water.
26/6	1100	6.4	8.32	0.232	83	2	
29/6	780	5.4	11.80	0.520	89	2	
30/6	890	5.1	11.92	0.785	79	2	Peroral intake of 5 g H_3PO_4 + 10 g NaH_2PO_4. 2 H_2O dissolv in 500 ml water.
1/7	960	5.4	10.90	0.615	103	2	
2/7	910	5.6	9.95	0.560	90	2	Peroral intake of 8 g glutamic a
3/7	1030	5.6	9.01	0.570	87	2	

Table VI b

K. T. 22 years, male.

Date	Diuresis	pH	Total-nitrogen	Ammonia-nitrogen	Glutamine	Glutamic acid	Remarks
1953 16/6	1050 ml	6.0	11.42 g	0.535 g	73 mg	2 mg	
17/6	1530	5.5	12.00	0.670	66	3	
18/6	1200	5.7	11.40	0.505	91	2	
19/6	1540	5.6	13.31	0.522	81	6	
22/6	990	6.2	9.15	0.346	97	2	
23/6	1770	6.4	10.20	0.363	98	3	Increasing the diuresis by drink 2 l water.
24/6	1200	6.6	9.75	0.287	82	3	
25/6	1500	7.4	13.40	0.179	97	6	Peroral intake of 10 g $NaHCO_3$ dissolved in 500 ml water.
26/6	910	6.6	7.85	0.242	78	4	
29/6	1000	5.4	14.70	0.885	86	2	
30/6	1290	5.2	14.10	1.030	103	2	Peroral intake of 5 g H_3PO_4+ 10 g NaH_2PO_4. 2 H_2O dissol in 500 ml water.
1/7	1360	5.8	12.42	0.640	112	3	
2/7	1700	5.8	10.40	0.605	89	3	Peroral intake of 8 g glutamic a
3/7	940	5.8	8.85	0.553	78	2	

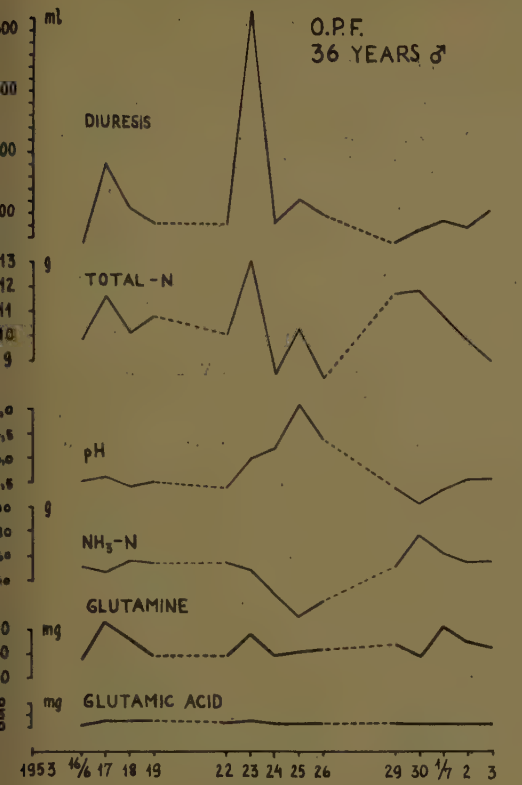


Fig. 1.

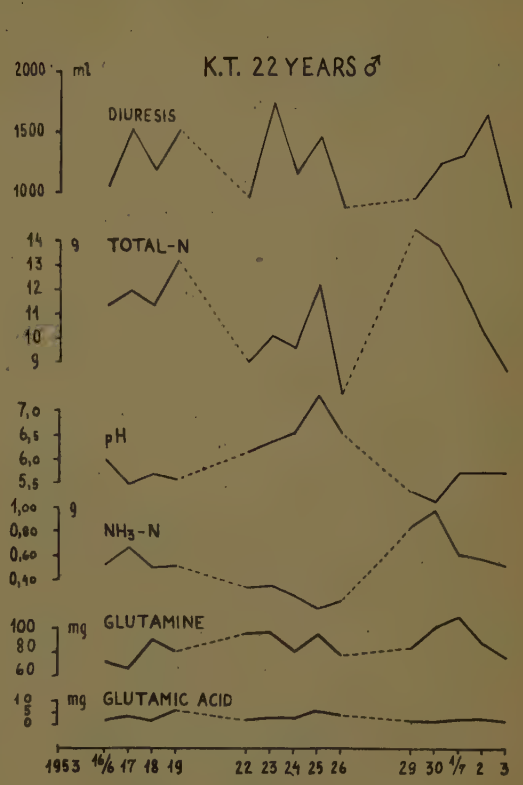


Fig. 2.

Fig. 1 and 2. The daily urinary excretion of glutamine and glutamic acid in two normal males during a period of about two weeks. Compared with the diuresis, the pH of the urine and the amounts of total-nitrogen and ammonia-nitrogen excreted in urine. See also Tables VI a and VI b.

At last the influence of peroral intake of utamic acid on empty stomach was instigated.

The results are shown in the following bles and diagrams.

The suspensions of Clostridium Welchii SR 12

The author has described the technic about e cultivation and production of Clostridium elchii SR 12 before (1952). The washed cterial cells were suspended in 0.2 M acetate buffer pH 4.9, shaken in vacuo in order to remove carbon dioxide and stored in frozen state. With such suspensions, containing 2—3 per cent of bacteria (dry weight) and vigorously shaken before use, the decarboxylation of glutamine and glutamic acid was carried out within 15 to 20 minutes at 40° C.

The specificity of the bacteria always was controlled against glutamine, glutamic acid, asparagine, aspartic acid, arginine and glycine. Further on, it was controlled that

no change in pressure was obtained when mixing the bacterial suspension with acetate buffer pH 4.9 0.2 M.

Accuracy

The pressure on each Warburg manometer may be read with an accuracy of approximately ± 0.25 mm. Each reading must be corrected with that on the combined blank—thermobarometer. Thus the combined inaccuracy for pressure reading will be approximately ± 0.5 mm.

By using Warburg vessels of about 15 ml volume and 3.2 ml urine in each determination, 0.5 mm increase in pressure corresponds to about 0.15 mg glutamine or glutamic acid pr. 100 ml urine. Thus the accuracy in the determination of glutamic acid will be ± 0.15 mg pr. 100 ml urine (± 1.5 mg pr. 1000 ml urine). In the determination of the sum of glutamine and glutamic acid the accuracy will be the same. By the calculation of glutamine, however, it will be ± 0.3 mg pr. 100 ml urine (± 3 mg pr. 1000 ml urine).

CONCLUSION

The excretion of glutamine and glutami acid was found to be relatively constant an mostly independent of variations in the diu resis, the pH of the urine and the amount of total-nitrogen and ammonia-nitrogen ex creted. Alkalinizing or acidifying the orga nism did not influence the excretion o glutamine and glutamic acid.

The amount of glutamine daily excrete was found to range between 66 and 112 mg that of glutamic acid between 1 and 6 mg

The excretion of these two substance seemed not to be influenced by peroral in take of 8 g glutamic acid on empty stomach

SUMMARY

A new method for the quantitative dete mination of glutamine and glutamic acid ha been described. With this method the ex cretion of these two substances in urine ha been followed up for about two weeks two normal males. The daily excretion glutamine was found to range between 6 and 112 mg, that of glutamic acid betwe 1 and 6 mg.

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METHOD FOR THE DETERMINATION OF THE GLOMERULAR FILTRATION RATE IN PATIENTS WITH URETEROCOLIC ANASTOMOSIS[1]

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As the glomerular filtration rate has not eviously been determined in patients with eterocolic anastomosis, a method will be scribed in the following which has been borated for the purpose of controlling e late results after the operation.

In determination of the clearance we nerally measure the amount of urine ex-eted during definite periods and the con-ntration of the substance in question. his procedure cannot be practised in pa-nts with ureterocolic anastomosis, partly cause it is impossible by simple collection measure the amount of urine excreted ring definite periods, and partly because e urine may change its composition in the urse of its passage through the colon.

Collection of urine through indwelling heters in the ureters is theoretically pos-le, but the introduction of a catheter rough the sigmoid would cause technical fficulties and involve a risk of infection. rthermore, collection of urine from the eter through an indwelling catheter is not antitative, even in catheterization through normal ureteral ostium.

However, in the methods generally employed for clearance determinations the chief error is also inaccurate collection of the urine, and it has therefore previously been attempted in different ways to avoid a direct measurement of the amount of the substance excreted in the urine.

Previous methods

It has been attempted to calculate the clearance from the slope of the curve indicating the fall in the plasma concentrations after a single injection of a subtance which is not converted or deposited in the organism. Barnett (1940) used inulin, Newman, Bordley & Winternitz (1944) mannitol, and Landowne & Alving (1947) para-aminohippuric acid. The calculation includes the volume of distribution determined by extrapolation, and it is a necessary condition that the distribution is even, and that the plasma concentration falls as an exponential function of the time. The method has afforded acceptable results in practice, but suffers from the defect that the necessary conditions mentioned cannot be realized to the fullest extent by the single injection technique (Schachter, Freinkel & Schwartz 1951, Robson, Ferguson, Olbrich & Stewart 1950 and Smith 1951).

[1] Aided by grants from King Christian X's ndation, Weimann's Bequest and P. A. Brandt's quest.

Earle & Berliner (1946) suggested a method based on the following principle: If a substance that is excreted only in the urine and not converted or deposited in the organism is injected at a constant rate, a constant plasma level must be established when the distribution has become complete; the amount of the substance injected per time unit will then be equal to the amount excreted through the kidneys per time unit. The clearance can then be calculated from the formula $C = IV/P$ where IV is the amount injected per minute and P is the plasma concentration.

Both with inulin and with para-aminohippuric acid the authors found conformity in 4 normal subjects between the results of clearance determinations performed simultaneously according to the principle mentioned above and according to the classic method with collection of the urine.

The duration of the experiment was about 90 minutes, and the dosage was calculated so that an initial injection of inulin would give a plasma concentration of about 5 mg per 100 ml; it was then attempted to maintain this concentration by constant infusion of a sustaining dosage calculated from the expected filtration rate.

Berger, Farber & Earle jr. (1948) used the same method for inulin clearance and PAH-Tm determinations and, on comparison with the results obtained by collection of the urine, found a standard deviation of $\pm$ 10 per cent. A constant plasma level was said to be found in most cases after an hour, but even when the experiments were extended to 2 1/2 hours, variations still occurred. The initial dose was always 2 g of inulin; this gave a plasma concentration of 10—15 mg per 100 ml, which was then attempted to be maintained by a sustaining dosage adapted to the expected filtration rate. With filtration rates less than 60 per cent of the normal, the method was found unsuitable.

The conformity with the results obtained by collection of the urine shows that the principle of constant infusion can be used, but several objections could justly be raised against the method in its original form. Robson, Ferguson, Olbrich & Stewart (1950) thus pointed out that in the case of the inulin clearance, the dosage required advance knowledge of the filtration rate. further the principle implied that a constant plasma level meant a constant amount of inulin in the organism, but that variations in the amount of inulin in the volume of distribution could not be recognized. These objections are especially justifiable when relatively short experimental periods are used as in the studies mentioned. In determination of the inulin volume of distribution, Guadino Schwartz & Levitt (1948) found that complete equilibrium was not established in human subjects until after at least 6 hours constant infusion.

Basis of dosage administered by the write

A further development of the method of constant infusion will be described in the following. It differs from the method mentioned above in that no attempt has been made to maintain the same plasma level with varying filtration rates by means of different sustaining dosages; only a constant plasma level is aimed at, and this is determined by the filtration rate and a fixed sustaining dosage.

n excretion through the kidneys of a stant amount of inulin per minute which als the amount administered per minute infusion (IV) is obtained by a plasma centration (P) which is determined by filtration rate (F) and inversely propor-

al to this, as $F = \frac{IV}{P}$, or $P \times F = IV =$

stant.

f a plasma level is to be maintained by ns of the same sustaining dose in per- s with different filtration rates, the initial ma concentrations must be in inverse o to the filtration rates.

n order to find out at what time after a le injection P and F were inversely pro- tional in 6 subjects with different filtra- rates, 10 g of inulin were injected intra- ously in the course of 20 minutes, and plasma concentrations were determined ntervals of 40 minutes during the next ours; the filtration rates were then cal- ted after collection of urine for 2 periods. was then possible to plot the curves ving the variations in $P \times F$ in the rse of 3 hours; it appeared that $P \times F$ e equalized in 160—180 minutes, indicat- that P and F were inversely proportional his time (Fig. 1).

his means that the constant sustaining sion should be commenced 160—180 utes after the priming injection.

Vith the dose of 10 g the plasma concen- on was found between 47 and 13 mg 100 ml after 170 minutes with filtration s varying from 35 to 123 ml/min. As optimal accuracy of the method of ana- is found at concentrations higher than t 10 mg per 100 ml, the concentrations

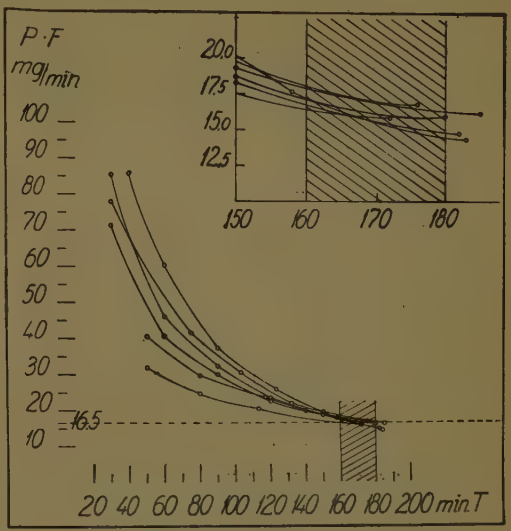


Fig. 1. Variations in the product plasma concentration (P) × filtration rate (F) after a single injection of 10 g of inulin in 6 subjects with different filtration rates. P and F became inversely proportional in 160—180 minutes.

found up to 170 minutes are of the order of magnitude desired, and 10 g of inulin must therefore be considered a suitable priming dose.

The sustaining dose to be used to maintain the plasma concentrations found after 170 minutes is determined by the value of $P \times F$, about 16.50 mg/min. at this time. As $P \times F = IV$, the dose must also be 16.50 mg/min., or about 1 g/hr, when equilibrium has been established.

After the single injection, maintenance of the plasma concentration for some time with the constant infusion is necessary in order that the difference in concentration between plasma and the remaining volume of distribution may be equalized. Differences either in the size of the volume of

distribution or in the rate at which inulin is distributed in different individuals may also give rise to deviation to be equalized during the sustaining period.

Method in control experiments

The filtration rate was determined by constant infusion in patients with normal or reduced renal function with simultaneous collection of urine as control. The experiments were performed as follows:

Table 1.

Time (minutes)	Intravenous infusion	Blood sample	Collection of urine
0		blank	
	10 g of inulin in 300 ml		
20			
	0.9 per cent saline, about 100 ml		
170		1	
240		2	
	1 g inulin/hr		I
280		3	
			II
320		4	
			III
360		5	

The procedure lasts 6 hours. After a blank blood sample has been taken, the patient is given a light breakfast. To secure an output of urine sufficient for voluntary evacuation of the bladder, the patient drinks 1 litre of water in the course of the last 4 hours of the experiment.

The times when the bladder is evacuated are recorded to the minute when for practical reasons they do not follow the schedule precisely.

For the reasons previously mentioned, the continuous infusion is started after 170

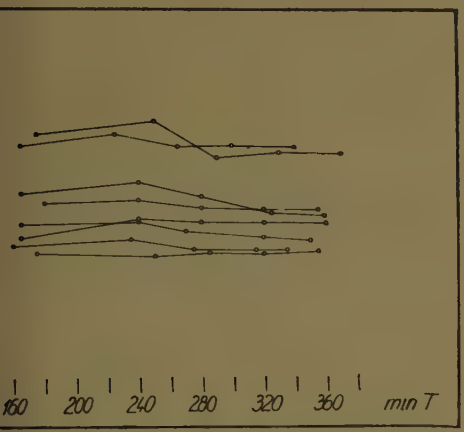
Fig. 2. The apparatus used for constant infusi

minutes and is effected with an infusi apparatus which, at a constant rate of ml/hr, empties a 100 ml record syringe w a 5 per cent solution of inulin. The inu was determined with the modification of diphenylamine-method described by Boje (1952). Duplicate determinations were ways made. The exact amount of inulin fused per minute was determined by analy in each experiment.

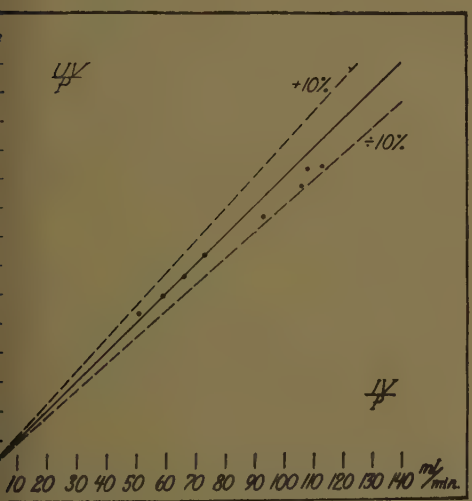
In order to settle the question as whether reabsorption of inulin from intestine is a source of error when method is used in patients with ureteroc anastomosis, the following experiment performed in 2 subjects:

Three blood samples were taken at int vals of 1 hour, after which 15 g of in dissolved in 250 ml of a 0.9 per cent N solution were administered by rectum. Bl samples were taken once an hour fo hours, and the urine produced in the cou of this period was collected.

All blood samples were analysed for cose and inulin, and the inulin values w corrected for changes in the blood-su level. The urine was analysed for inulin an portion of urine passed before the adm stration of inulin and diluted to the s



3. The inulin concentration in plasma in the se of the constant infusion of 1 g of inulin/hr.



4. The inulin clearance determined by infusion $\left(\frac{IV}{P}\right)$ and by collection of urine $\left(\frac{UV}{P}\right)$ in 8 subjects with different filtration rates.

r as the sample was used as a blank nese cases.

n all experiments, penicillin has been inistered simultaneously with inulin in order to examine the suitability of the former for constant infusion clearance, etc. The results of this part of the investigation will be reported later.

Results of control experiments

The filtration rate was determined in patients with normal or reduced filtration rates and, as already mentioned, 5 blood samples were taken at intervals in the course of the constant infusion. It is apparent from Fig. 3 that the plasma concentration present 170 minutes after the injection of a priming dose of 10 g of inulin was maintained by the sustaining dose of 1 g of inulin/hr.

In calculating the filtration rate on the basis of the amount of inulin excreted in the urine, the mean figures of the plasma concentrations before and after each of the 3 periods of collection have been used. In the calculation from the quantity infused per minute the last of the measured plasma concentrations was used.

The conformity between the results calculated by infusion $\left(\frac{IV}{P}\right)$ and by collection of urine $\left(\frac{UV}{P}\right)$ is shown in the diagram above. The deviations did not exceed ± 10 per cent (Fig. 4).

In the experiments with rectal administration of 15 g of inulin, this substance could not be demonstrated in the blood within 6 hours, and less than 0.10 per cent of the quantity administered was found in the urine produced in the course of the experiment.

DISCUSSION

The advantage of this method is that determinations of the filtration rate without collection of the urine can be carried out with the same dosage of inulin over a wide range of filtration rates.

The constant infusion is not started until the equalization between the plasma and the remaining extracellular space is advanced and, as the plasma concentration shows good constancy in the sustaining period, the plasma concentration at the end of the experiment must be supposed to be very near the average concentration in the total extracellular space.

It is not necessary to have any advance knowledge of the filtration rate, but it must be pointed out that the dosage stated only permits determination of filtration rates down to about 30 ml/min. With lower filtration rates the total amount of inulin injected would not suffice to establish the concentration required in the volume of distribution. In such circumstances the plasma concentration will tend to increase, and a constant level will not be obtained within the limit time of the experiments.

The length of the infusion period is apparently sufficient to equalize such variations in the inulin concentration in the plasma after 170 minutes as might be caused by moderate differences in the size of the extracellular space, but it should be mentioned that the standard dosage cannot be used in cases of ascites.

Broadly speaking, the plasma levels remained constant during the maintenance infusion, as has already been mentioned. However, in most cases there is a slight rise in the beginning owing to the fact that concentration in the intercellular fluid the time the infusion is started is hig than the plasma concentration (Schach Freinkel & Schwartz, 1950), so that further equalization will commence wit rise in the plasma concentration.

It might seem tempting to omit timewasting constant infusion and de mine the plasma concentration at the t when the infusion should have been c menced. However, such a simplified pr dure cannot be recommended, as differe in the volume of distribution and in the r at which inulin is distributed in differ individuals might lead to uncontrolla errors.

The experiments with rectal administ tion seem to exclude the possibility that absorption of inulin from the intestine n influence the plasma concentration when method is used in patients with ureteroc anastomosis.

SUMMARY

The paper describes a modification of constant infusion method in which filtrat rates as low as 25 per cent of the nor can be measured with the same dosage inulin and without collecting the urine.

The theoretical basis is reviewed, an comparison is made with the results ca lated on the basis of collection of urin

With a view to the applicability of method in patients with ureterocolic a stomosis, it has been ascertained that absorption of inulin from the intestine not take place to an extent involving errors.

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THE EFFECT OF AN ANION EXCHANGE RESIN ON PEPSIN

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INTRODUCTION

The importance of pepsin in the pathogenesis of peptic ulcer

The causes of peptic ulcer are still unknown. Two of the main factors are, according to Ivy, Grossman & Bachrach, 1950:

a) The mucous membrane factor, causing a locus minoris resistentiae.

b) The pepsin-HCl-factor which may develop autolysis. There are two chemical factors in the gastric juice of importance for autolysis:

1) HCl, which may act directly on the mucous membrane or indirectly via its pepsin activating ability.

2) Proteolytic enzymes. Rennin probably is not present in adults (Cantarow & Trumper, 1949). Kathepsin has its optimum at pH 4—5 (Evans, 1945), and probably is of no importance. There remains only pepsin with its optimum at pH 1—2 (Northrop, Kunitz & Herriott, 1948).

Several experimental data indicate the importance of pepsin in the pathogenesis of peptic ulcer. Thus LeVeen (1947) maintains that HCl has only an indirect effect. A weak solution of HCl with a high pepsin activity produced more frequent and larger ulcer than a strong solution with a low pepsi activity. Any therapy which reduces th peptic activity should be of interest.

Ion exchange resins

Antacida usually have no other effect tha that of neutralizing the acid (Warren, Fro & Kirsner, 1943). With the anion exchang resins a new principle has been introduce in the therapy of peptic ulcers. The use o ion exchange resins was suggested in 194 by Skogseid (1951), and introduced b Segal, Hodge, Watson & Scott in 194 Segal *et al.* used Amberlite IR-4. They mai tain that this resin has no direct effect o pepsin, but has an indirect inactivating effe by increasing the pH.

In a later publication it is demonstrate that Amberlite IR-4 inactivates 80 per ce of the pepsin at pH 1 (n/10 HCl). Gre quantities of the resin were used (3 g t 100 mg of pepsin). Trypsin was inactivate in the same manner, and this inactivatio was irreversible, but it is not mentione whether this also applies to pepsin (Marti & Wilkinson, 1946).

At Rikshospitalets Medisinske Poliklinik Oslo, good results have been obtained wit an ion exchange resin in the treatment o peptic ulcer (Dedichen, 1952).

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[1] With the technical assistance of Miss M. Pedersen.

The object of the present investigation s been to study the mode of action of the ion exchange resin on pepsin-HCl solu-ns and on gastric juice in vitro. It has en attempted to answer the following estions: 1. Is the pepsin affected by the ion exchange resin? 2. To what degree? In which way?

he general properties of the anion ex-change resin

The anion exchange resin used in this udy was polyaminostyrene:

t is manufactured by Norsk Hydro-Elek-sk Kvælstofaktieselskab (Skogseid, 1948, 51), and is compounded and distributed Nyegaard & Co A/S under the trade me Macrin "Nyco".)

The brownish material is insoluble in all e usual solvents. It is an anion exchange sin, a weak base which give salts with rong acids. The salts are also insoluble in ater.

The experiments have been carried out th a commercial preparation from the me batch without further purification. It s been screened according to the standards Ph. N. 5th Ed. 1939.

METHODS

For the pepsin determinations Hunt's method s been used with dried, human plasma as sub-rate (Hunt, 1948). The readings were made in Spekker photoelectric absorption meter (Hilger) d for some analyses in a Beckman spectrophoto-

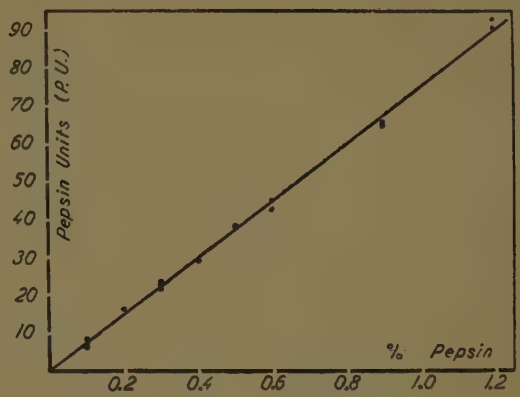


Fig. 1. Accuracy of the estimation of pepsin by Hunt's method.

meter. All analyses have been made in duplicate with a few exceptions which are mentioned in the text. Hunt's standard is used, and the pepsin activity is given in Pepsin Units (P. U.) according to Hunt's definition.

Hunt states the error to be ± 2.64 per cent, and Linde has found ± 4 P. U./ml (Linde, 1950). Such calculations of error are not made in this work, but the diagram in Fig. 1 is an expression of the usefulness of the method (no measurements are omitted).

The pH determinations were made with a glass electrode in a Cambridge portable pH-meter. The error of measurement is ± 0.05.

For the experiments with pepsin, Pepsinum concentratum (Langebeck) 1 : 2000 was used. (For the greater part of the experiment B. 4. a different and apparently stronger portion was used.) Filtration reduces the pepsin activity, all analyses were therefore filtered in the same way.

The stirring was done in a glass beaker with an electric agitator.

The diagrams illustrate the results of representative single experiments, and are not constructed as statistical average curves.

EXPERIMENTS

A. *The acid binding capacity of Macrin*
Procedure: Macrin of 30 mesh in quantities from 0 to 5 per cent was added to

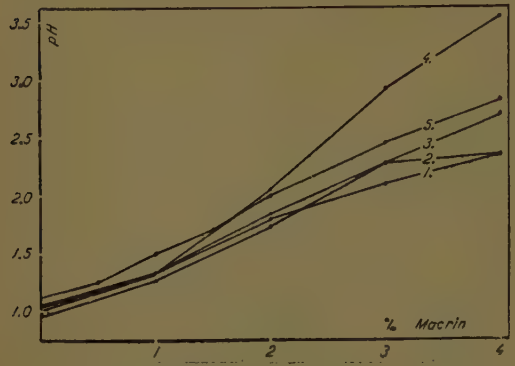


Fig. 2. The effect of Macrin on the pH.
1. HCl.
2. » with 1 mEq NaCl per L.
3. » » 10 » »
4. » » 100 » »
5. » » 1 per cent of pepsin.

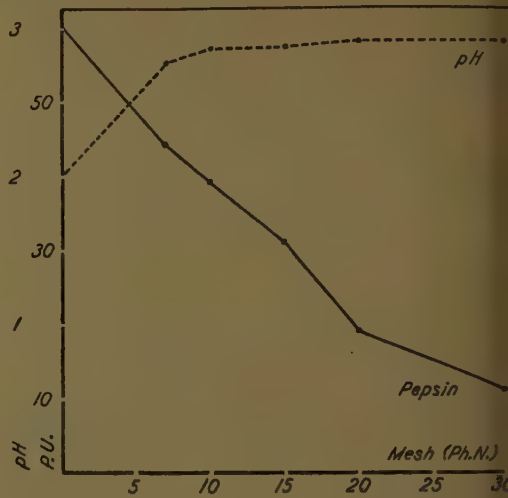


Fig. 3. The grain size of Macrin.

approximately n/10 HCl. It was stirred for 20 minutes, filtered, and pH was determined in the filtrate. The same test was made with the addition of 1 per cent pepsin and varying quantities of NaCl.

Results: See Fig. 2.

Comments: The increase in pH shows an approximately linear relationship to the quantity of Macrin (in the range which was investigated). With the addition of pepsin and NaCl the increase in pH was more marked.

B. *The pepsin-inactivating effect of Macrin (Pepsin-HCl solutions)*

1. *The importance of the grain size*

Procedure: A solution of 1 per cent pepsin in HCl was adjusted to pH 2.1. Macrin of decreasing grain sizes, in a quantity of 1 per cent weight, was added to aliquots. After stirring for 20 minutes and filtering, pH and pepsin were determined in the filtrate.

Results: See Fig. 3.

Comments: The inactivation of pepsin in creases when the total surface of the Macri is enlarged. The same holds true for th acid binding capacity. This is in accordanc with the observations of Martin & Wilkin son (1946). The relation is different fo pepsin and for pH. The inactivation o pepsin is approximately inversely propor tional to the grain size, whilst pH does n increase further when the grain size is re duced below a certain lower limit.

2. *The importance of the stirring time*

Procedure: The same pepsin-HCl solutio was used as in the previous experiment, an Macrin of 30 mesh was added in a quanti of 1 per cent. Aliquots were stirred fro 20 to 1 minutes, filtered immediately, an pepsin and pH were determined in th filtrate. The experiments were repeated wi a 1.5 per cent solution of pepsin with 1 per cent Macrin added. Test were also ma without the addition of Macrin.

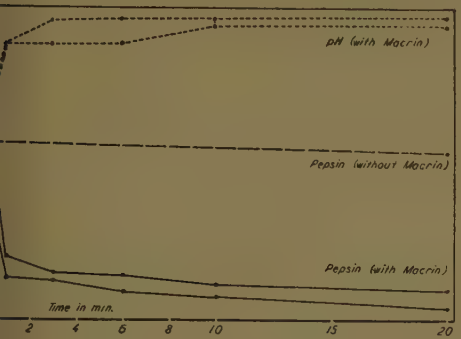


Fig. 4. The time of stirring.

lts: See Fig. 4.

ments: The inactivation of pepsin in- ses with the stirring time, but after the minute the increase is very slight. The reaches its maximum during the first ute.

he importance of the quantity of Macrin

edure: 1 and 2 per cent solutions of in in HCl were adjusted to pH 2.1. rin of 30 mesh was added to aliquots in tities varying from 0 to 2 per cent eight. The mixtures were stirred for 20 utes, filtered, and pH and pepsin were rmined in the filtrates.

lts: See Fig. 5.

ments: The inactivation of pepsin r these circumstances is approximately ortional to the quantity of Macrin.

ow does Macrin inactivate the pepsin?

acrin may only have an indirect effect he pepsin, caused by the increase in the or it may act directly on the pepsin. nswer this question, the relation between pepsin activity and the pH was first stigated.

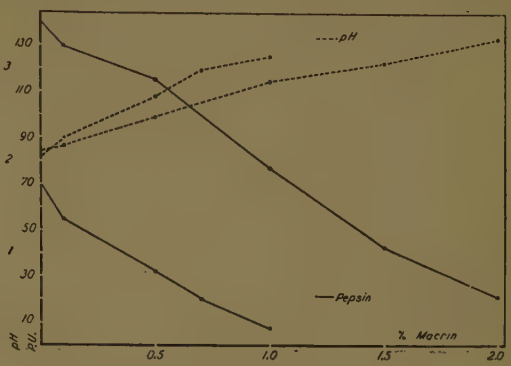


Fig. 5. The quantity of Macrin.

Procedure: 1 per cent pepsin solutions, with pH ranging from 1 to 4, were made, kept in refrigerator over night, and then the pepsin was determined. The pH of the substrate was adjusted in accordance with the pepsin solutions.[1] — The experiment was repeated, the pH of the substrate being constant at 2.1, the pH of the pepsin solutions ranging from 1 to 7. These tests were not made in duplicate.

Results: See Fig. 6.

Comments: The experiment with adjusted substrate shows the total reduction in peptic activity as the pH increases. This reduction is roughly proportional to the increase in the pH in the interval from pH 2 to pH 4. — The experiment with constant substrate shows the irreversible part of the inactivation. In the interval from pH 2 to pH 4 the inactivation is mainly reversible.

The pepsin inactivation of Macrin may therefore be ascribed to the increase of the

[1] The adjustment of the pH in the substrate in this and the following experiments is quite essential. It was suggested by the Research Division of the Glaxo Laboratories in England.

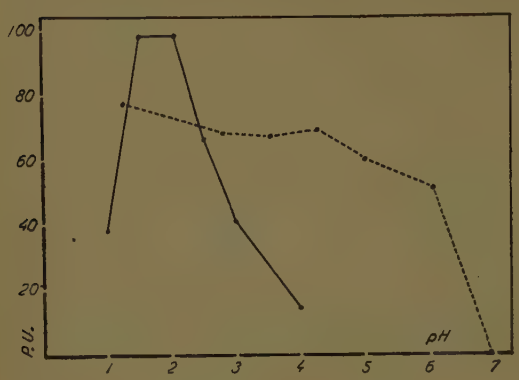


Fig. 6. The activity of pepsin at different pH.
— — — — pH of the substrate is constant.
———— pH of the substrate is adjusted.

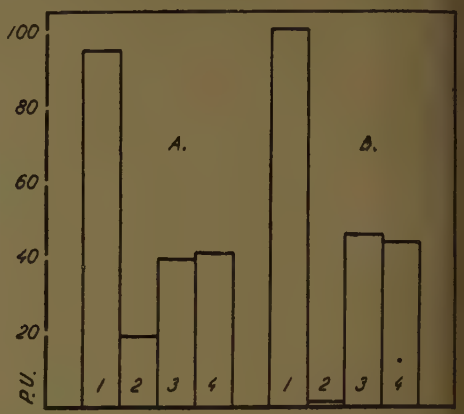


Fig. 7. The inactivation of pepsin with Macrin with NaOH.

		pH A
1.	Pepsin-HCl	2.03
2.	» and Macrin. Filtered....	3.00
3.	» and Macrin. Not filtered	3.00
4.	» and NaOH	3.00

Macrin added 1 %

pH. To settle this question, the effect of Macrin is compared to that of NaOH.

Procedure: A 1 per cent solution of pepsin in HCl was adjusted to pH 2. One portion (1) was filtered, pH and pepsin being determined in the filtrate. To another portion (2) 1 per cent of Macrin was added, the mixture stirred for 10 minutes, filtered, and pH and pepsin determined in the filtrate. The third portion (3) was filtered, 1 per cent of Macrin added, the mixture stirred for 10 minutes, and pH and pepsin determined without the Macrin being filtered off. The fourth portion (4) was adjusted to the pH of (2) and (3) with NaOH. The substrates had the same pH as the samples. The experiment was repeated, starting at pH 1.5, 2 per cent of Macrin being added.

Results: See Fig. 7.

Comments: Macrin clearly has a double effect:

1) One part of the pepsin inactivation is due to the increase of the pH. This effect is identical with that of alkali, and if the Macrin is not removed, this one is the s effect.

2) The other part is due to the absorpt of pepsin. The Macrin being filtered this effect is in part added to the first If it is not removed, the absorbed pep appears to have the same peptic activity free pepsin. The pepsin absorbed to Amb lite IR-4 also shows considerable peptic tivity (Wilkinson & Martin, 1946). At same pH the effect of absorption beco greater as increasing quantities of Ma are added.

5. *The influence of the pH on the pe absorption of Macrin*

Procedure: Two 1 per cent stock solut of pepsin were made, the pH adjuste 1 and 2. To aliquots of each solution,

ing quantities of 30 mesh Macrin were
l. Each sample was stirred for 20
tes, filtered, and pH and pepsin deter-
l in the filtrate. These tests were not
in duplicate.

lts: See Fig. 8.

ments: In the solution originally ad-
l to pH 2, the pepsin activity decreased
ly about proportionally to the increas-
quantities of Macrin (see experiment
. In the solution starting at pH 1, how-
the pepsin activity did not decrease
edly until the pH had been increased
e 2. Then the curve is about parallel to
ne of the other solution.

The reversibility of the pepsin absorption of Macrin

edure: A 1 per cent pepsin solution
adjusted to pH 2.1. One portion (1)
filtered, and in the filtrate pH and
n were determined. To the rest, 1 per
of 30 mesh Macrin was added, and the
ure stirred for 20 minutes. One half of
portion (2) was filtered, pH and pepsin
determined in the filtrate. The other
(3) was brought down to pH 2.1 with
drops of 2 n HCl, after shaking settled
0 minutes at room temperature, filtered,
pH and pepsin were determined in the
te.

lts: See Fig. 9.

ments: In this pH interval the greater
of the pepsin could be reactivated with
ddition of HCl.

e pepsin was reactivated by lowering the
o the original value, approximately 2.
riment B. 5. suggested that the pepsin
not be reactivated until the pH had

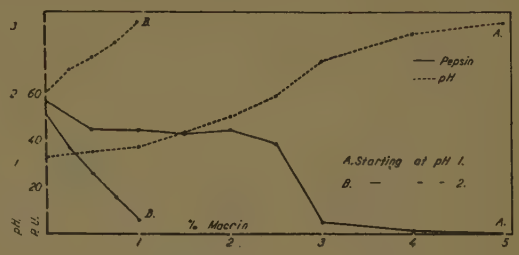


Fig. 8. The influence of pH on the pepsin absorption by Macrin.

Fig. 9. The reversibility of pepsin absorption by Macrin.

1. Pepsin-HCl (pH 2.10).
2. » and Macrin (pH 3.06).
3. » and Macrin. HCl added before filtering (pH 2.00).

come down into this region, even if the original solution had a higher pH. The following experiment should corroborate this statement:

Procedure: A 1 per cent pepsin solution in HCl was adjusted to pH about 3. One portion (1) was filtered, and pH and pepsin were determined in the filtrate. To the rest was added 1 per cent of 30 mesh Macrin which was stirred for 20 minutes. A small portion (2) was then filtered, and pH and pepsin were determined in the filtrate. The remainder was divided into four portions

(3 a, b, c, d). Each portion was brought down to the pH wanted, after shaking settled for 10 minutes, filtered, and pH and pepsin were determined in the filtrate.

Results: *See* Fig. 10.

Comments: Pepsin was not reactivated when the pH was lowered to the original value of about 3. The reactivation did not occur until the pH had come down to about 2.

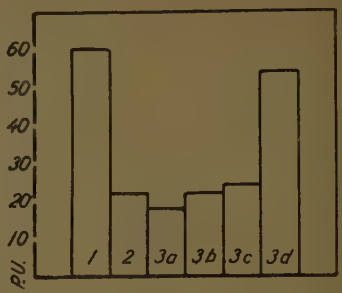


Fig. 10. The reversibility of pepsin absorpti by Macrin.

1. Pepsin-HCl (pH 2.80).
2. » and Macrin (pH 3.60).
3. » and Macrin. Increasing amount HCl added before filtering:
   a) pH 3.22
   b) » 2.88
   c) » 2.52
   d) » 2.00

C. *The effect of Macrin saturated with HCl*

These experiments should indicate whether the effect of Macrin changes when the active amino group is combined with HCl. The experiments were made with a Macrin preparation which had been saturated with HCl. (The usual commodity had been steeped in concentrated HCl, dried and sieved through a 30 mesh screen.)

Procedure: A 1 per cent pepsin solution in HCl was adjusted to pH 2.1. 1 per cent by weight of the preparation was added, the mixture stirred for one hour, filtered, and pH and pepsin were determined in the filtrate. Check tests were made without any admixture.

Results: See Fig. 11. The results of experiment B. 2 are included for comparison.

Comments: pH and pepsin both showed a small, but constant fall. The fall in pepsin activity was considerably less than that obtained with the usual Macrin. The effect could possibly be explained by the diffusion of HCl from Macrin-HCl out into the solution, the pepsin taking the vacant places. The pepsin inactivation roughly corresponds to the expected, if the fall in pH is taken as an

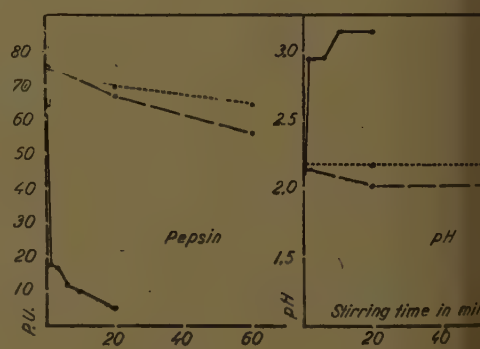
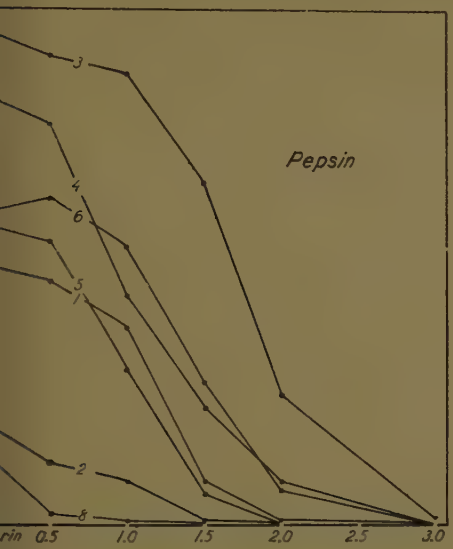


Fig. 11. The effect of Macrin, saturated with compared to the effect of Macrin.
..... Pepsin-HCl.
——— » and Macrin.
— — — » and Macrin, saturated wi HCl.

expression for the liberated Macrin experiment B. 3.) The results show tha active amino groups are necessary for inactivation of pepsin.



g. 12. The effect of Macrin on gastric juice.

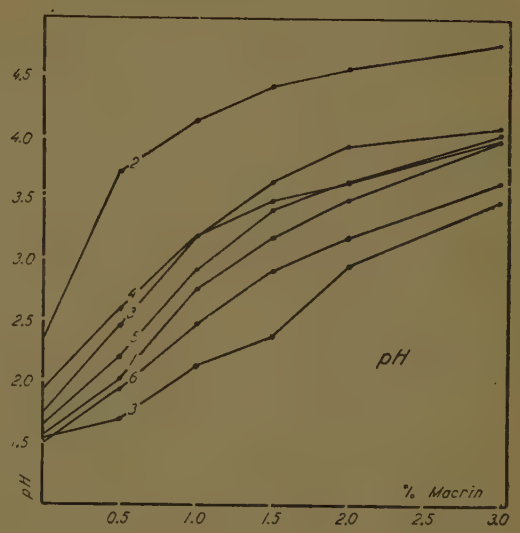


Fig. 13. The effect of Macrin on gastric juice.

The effect of Macrin on gastric juice

edure: The investigations were carried n the gastric juices of eight consecutive nts, on whom gastric aspirations were ormed for diagnostic reasons. The aspi- ns were performed in the morning, on mpty stomach. If less than 40 ml were ined, 2 ml 1/4 ‰ Histamine were given utaneously and the aspiration continued Table 1). The gastric juice was filtered, pH determined in the filtrate. Then l portions of HCl with pH 2.1 were d (cf. Hunt, 1948). Increasing quan- of 30 mesh Macrin were added to ots, the mixture stirred for 20 minutes, ed, and pH and pepsin were determined e filtrate. The pH in the substrate was tant at pH 2.1.

lts: See Figs. 12 and 13. The numbers ne curves correspond to the numbers in able.

Comments: The pH increased fairly uniformly, but did usually not exceed 5. Three per cent by weight of Macrin reduced the acidity to about 1/100 of its original value. The pepsin activity decreased. In the beginning the inactivation was small, but increased vigourously when the quantity of Macrin reached above a certain level, differing for each sample. Above this level, the pepsin activity fell evenly. The very weak gastric juices inactivated more slowly. The bend in the curves towards the end is probably artificial, caused by the fact that the pepsin determinations have not been made at adequately small intervals.

DISCUSSION

1. *The effect on the pH*

Macrin increases the pH in an acid solution. The acid binding capacity is approximately 6 mEq/g at pH below 2.

These experiments show that the pH continues to increase, in any case to 4, if the quantity of Macrin is adequately increased. The addition of pepsin and NaCl causes the pH to increase more quickly. In vitro the pH can be increased above the usual upper level in the stomach, but for this very high dosages of Macrin are needed, probably far above the usual dosage. (3 per cent of Macrin did, for instance, increase the pH from 1.5 to 3.5 in a gastric juice.) It is not known whether this is followed by a rebound in gastric acidity (Wirts & Rehfuss, 1950).

2. *The effect on pepsin*

Macrin inactivates pepsin, partly by increasing the pH, partly by absorption.

The effect of increasing the pH is studied in experiment B. 4. It starts at pH 2, and then proceeds about proportionally to the increase in the pH. It is identical with the effect of alkali, and is dependent only on the pH, not directly on the concentration of Macrin. It is mainly reversible. When the Macrin is not filtered off, this effect will be the only one.

The effect of absorption is studied separately in the experiments with a constant pH in the substrate. The effect of increasing the pH does not come into play under these experimental conditions.

Macrin seems to start its absorption of pepsin when the pH is increased to about 2, because:

1) In a pepsin-HCl solution the pepsin activity does not decrease markedly before the pH is increased to about 2 (experiment B. 5). The same seems to hold for the gastric juices (experiment D).

2) The pepsin can be reactivated fr the Macrin-pepsin complex if the pH lowered to approximately 2, whilst no activation takes place above this level (periment B. 6).

Skogseid has shown that: "By increas the pH (say above 2) an increasing num of the Macrin's amino groups will appea a free state" (Skogeid, 1951). Therefo the absorption of pepsin probably pres poses free amino groups. Note that Ma which was saturated with HCl had no ef (experiment C). The effect is therefore sheer physical absorption.

HCl probably has the first priority on Macrin. Above pH 2, the pepsin can disp of the free amino groups, and the absorpt then proceeds proportionally to the quan of Macrin. The absorption amounts approximately 60—70 P. U. for each cent of Macrin (experiment B. 3). In gastric juices the absorption is of the s order, when the pH is above 2 (exp ment D).

The effect of absorption may also studied as the difference between the t effect and the effect caused by the incr ing of the pH (experiment B. 4). In th experiments this effect appears less expected according to experiment B. 3, reason being as yet unrevealed. Above p it is not dependent on the pH, but on concentration of Macrin. Absorbed pe apparently has the same effect as free sin. The effect of absorption may there only be demonstrated when the Macri filtered off.

The absorption of pepsin is revers (experiment B. 6). No essential change therefore, take place in the pepsin mole

ıg the absorption. This corresponds the fact that the Macrin-pepsin com- seems to have the same peptic activity ee pepsin (experiment B. 4).

ıe inactivation of pepsin is firstly de- ent on the pH and the dosage of Ma- secondly it is dependent on the Macrin g filtered off. When the Macrin is not ed off, the effect is that of alkali. On ing off, the effect becomes greater vided the removal takes place before the is decreased to such a degree that the in is eluted). It is a matter of opinion her the calculation of dosage in vivo ld be based on the supposition that rin is removed. Sooner or later the rin is carried out of the stomach. Therefore it seems justified to base the calculation on the supposition that Macrin, at least partly, is removed. In spite of this, the theoretical dosage necessarily has to be fairly high, far above the usual clinical dosage, but such an estimate will always be encumbered with a great factor of uncertainty, and will therefore be of minor value. In practice the effective dosage varies within wide limits. With Amberlite IR-4 the dosage varied between 0.5 and 102 g per 24 hours, with an average of 24 g (Spears & Pfeiffer, 1947). The point is that Macrin can inactivate the gastric juice, and the supplied quantity may be adequate. This has to be determined by clinical experience, and the dosage can only be fixed empirically.

Table I. *Survey of the gastric juices which have been investigated*

No.	Age	Sex	Diagnosis	Histamine given	pH in pure gastric juice	P.U.
1	29	♂	Obstipatio spastica	+	1.35	85
2	32	♂	Gastritis	+	2.59	35
3	35	♂	Ulcus duod.	+	1.22	162
4	43	♀	Anemia sideropenica	—	1.70	141
5	21	♂	Gastritis	—	1.44	98
6	43	♂	Dyspepsia	+	1.22	100
7	19	♂	Obstipatio spastica	—	2.86	1
8	57	♀	Dyspepsia	+	1.62	26

CONCLUSIONS AND SUMMARY

Polyaminostyrene (Macrin) increases pH in a hydrochloric acid solution oximately proportionally to the quantity Macrin. This has been investigated for pH range from 1 to 4.

Macrin can inactivate pepsin:

The effect increases with decreasing n size. The mode of action is here some- different for the pH and for pepsin.

b) The effect takes place quickly, mainly within a minute.

c) The effect does not start before the pH is above about 2.

d) The effect is a double one. Firstly Macrin reduces the peptic activity by increasing the pH, secondly by absorbing pepsin. When the Macrin is filtered off, both effects can be demonstrated. If it is not removed, the effect is identical with that of

alkali. The absorbed pepsin therefore apparently has the same activity as free pepsin.

e) The effect is mostly reversible.

f) Macrin which has been saturated with HCl is practically inactive, thus it can not be assumed that Macrin acts by physical absorption.

3. In gastric juice Macrin increases the pH and absorbs pepsin in the same manner as in the pepsin-HCl solutions. Its clinical use in peptic ulcer seems therefore theoretically well founded. The question of dosage has to be settled by clinical trial.

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VITAMIN K AS ANTAGONIST TO ANTICOAGULANTS

THE EFFECT OF SYNTHETIC VITAMIN K (MENADIONE) AND VITAMIN K_1 IN HYPOPROTHROMBINEMIA INDUCED BY DICOUMAROL, TROMEXAN AND PHENYLINDANEDIONE[1]

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n addition to dicoumarol, which has been l for clinical purposes for about 10 years, umber of other substances of similar ct have come into use during recent rs; especially tromexan and phenylin-edione (P.I.D.) have been put to prac-l use. It is a well-established fact that min K is able to counteract hypopro-mbinemia induced by dicoumarol, where-the view of the antagonistic effect of min K on tromexan and P.I.D. has ed. Bronstein & Witkind (1951) found istinct effect of synthetic vitamin K on nexan, whereas Douglas & Brown (1952), hey (1952), and Scarone, Beck & ight (1952) found little or no effect. h regard to P.I.D., Stohlmann & Smith 44), Jaques, Taylor & Lepp (1949) and ues, Gordon & Lepp (1950), were un- to observe an antagonistic effect of thetic vitamin K in animal experiments; same negative result was found by gien & Solier (1948) and Toohey (1952) xperiments with human subjects. Bjerke-l (1950) observed an effect of synthetic min K on P.I.D. after intravenous in-ion, while the results after administra- by mouth were doubtful.

Aided by a grant from King Christian X's ndation.

During recent years the fat-soluble vitamin K, vitamin K_1, has been used to an increasing extent as an antagonist to anticoagulants, especially in cases of severe intoxication. Stragnell (1952) thus found that it exerted a pronounced effect on dicoumarol; Scarrone *et al.* (1952) observed its effect on tromexan, Douglas & Brown (1952) found that it was effective on both dicoumarol and tromexan, and Toohey (1952) observed the effect on dicoumarol, tromexan and P. I. D. In the two last-mentioned studies a direct comparison was made between the effect of K_1 and that of the synthetic vitamin K, and the former was found to be far superior. There are no reports on failing effect of the fat-soluble vitamin-K. It appears from the litterature that these observations have led to a pronounced tendency to use K_1 in all cases where it is desired to counteract the effect of anticoagulants; as will be mentioned later, we do not consider this to be justifiable as a matter of course.

The purpose of the present study was primarily to examine the effect of the synthetic vitamin K, Menadoine, on dicoumarol, tromexan and P.I.D. under such uniform conditions that a comparison of the effect on these three substances was possible.

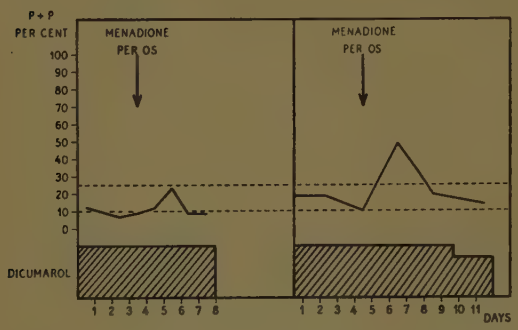

Fig. 1. Two examples of the effect of menadione on the P & P value. In these two cases, menadione was administered by mouth. In the first case the effect was very moderate; in the second it was rather pronounced.

Moreover, the effect of vitamin K_1 was examined in order to obtain an impression of the action of this substance in comparison with that of Menadione.

METHODS

The examinations were made on patients undergoing treatment with anticoagulants in a medical department; vitamin K was administered at a time when the condition of the patients did not contraindicate the resulting rises in the prothrombin concentration.

The anticoagulants employed were dicoumarol, tromexan in the form of the Czecho-Slovakian preparation "Trombex", and phenylindanedione in the form of the English preparation "Dindevan". Before the vitamin-K experiments were made, it was made sure that the prothrombin concentrations were at fairly constant levels within the therapeutical interval, i. e. between 10 and 25 per cent. The vitamins of the K-group were administered as follows:

1) 100 mg of menadione by mouth in the form of 10 tablets of 10 mg.
2) 100 mg of menadione intramuscularly in the form of 5 ml of a 2 per cent solution.
3) 100 mg of vitamin K_1 by mouth in the form of 2 ml of a 5 per cent alcoholic solution, emulsified in about 10 ml of water.

Administration of the maintenance dose of t anticoagulant was continued while vitamin K w administered. The effect of the vitamin was es mated from the rise in the prothrombin concentr tion. The maximal prothrombin concentration a the time when it was ascertained were recorde in addition it was observed for how long a peri the rise lasted. Figure 1 shows two examples fr the experiments with the effect of menadione adm nistered by mouth.

The prothrombin determinations were ma according to Owren's P & P method; when prothrombin concentration is mentioned in t paper, it thus covers the total prothrombin-proc vertin content.

RESULTS

The effect of menadione administered mouth appears from Table I. It shows th the effect on the three anticoagulants us was alike. It is highly varyable from o patient to another.

Table II shows the effect of menadio in intramuscular injection. Here, too, a u form effect was observed on the three su stances; the individual variation was pronounced as in peroral administration.

A comparison between Tables I and shows that the effect of menadione is ali whether it is administered by mouth intramuscularly; this applies both to t maximal prothrombin concentration, t time when it occurs and the duration the rise.

The effect of vitamin K_1 administered mouth is shown in Table III. The effect the three anticoagulants was alike. It w pronounced in most cases, though modera or slight in a few. An example hereof shown in Fig. 2; the patient's response menadione administered by mouth w typical, whereas it was very slight to pero administration of vitamin K_1 in two exa

able I. *Effect of menadione, 100 mg by mouth, on the P & P value in patients treated with dicoumarol, tromexan or P.I.D.*

	Patients	Mean initial value P&P per cent	Increase in P&P value		Days before maximal P&P value		Duration of effect days		Maintenance dosage mg	
			Range	Mean	Range	Mean	Range	Mean	Range	Mean
umarol	8	13	3–53	26	2–3	2.1	3–4	3.7	40–120	68
mexan	8	21	3–43	21	1–4	2.0	2–7	4.3	300–600	504
D.	8	12	5–64	24	1–3	2.0	3–6	3.8	50–100	75

able II. *Effect of menadione, 100 mg intramuscularly, on the P&P value in patients treated with dicoumarol, tromexan or P.I.D.*

	Patients	Mean initial value P&P per cent	Increase in P&P value		Days before maximal P&P value		Duration of effect days		Maintenance dosage mg	
			Range	Mean	Range	Mean	Range	Mean	Range	Mean
umarol	8	9	5–39	20	1–4	1.9	2–4	3.3	40–80	68
mexan	8	17	10–54	27	1–3	1.9	2–6	3.9	300–900	581
D.	8	11	5–55	20	1–3	1.9	2–6	3.7	75–100	81

ions. Therefore the usual dose of vitamin was given in intravenous injection, and exerted a pronounced effect. In this case slight effect of peroral administration of min K_1 has apparently been due to detive absorption from the intestinal canal. Finally, it appears from the tables that min K_1 exerts a more vigorous effect, ich perhaps sets on a little sooner and s somewhat longer than that of menane.

DISCUSSION

We have found that menadione both in roral and intramuscular administration rted an effect which was alike on dicourol, tromexan and P.I.D. The effect was highly varying from one patient to another; in a few cases little or no effect was observed. The reason why certain authors — as mentioned in the introduction — have found no effect of menadione on one or more of the anticoagulants mentioned is in some cases that small doses of the substance have been used. In other cases the administration of anticoagulant has been discontinued when menadione was given; this, of course, will tend to slur the effect of menadione, especially on tromexan and P.I.D. the effect of which soon subsides. The use of a relatively large dose of menadione in conjunction with the continued administration of the maintenance dose of the anticoagulant is, there-

Table III. *Effect of vitamin K1, 100 mg by mouth, on the P & P value in patients treated with dicoumarol, tromexan or P.I.D.*

	Patient	Initial value P&P per cent	Increase in P&P value	Days before maximal P&P value	Duration of effect days	Maintenance dosage mg
Dicoumarol..	1	18	60	2	10	60
	2	22	63	2	21	80
	3	8	13	1	4	100
	4	10	40	2	13	40
	5	6	22	1	4	60
	Mean	13	40	1.6	10.4	68
Tromexan ..	1	10	75	2	—*)	600
	2	24	52	2	9	600
	3	17	51	1	7	600
	4	6	62	1	6	600
	5	7	43	1	4	600
	Mean	13	57	1.4	6.5	600
P.I.D.	1	18	82	2	—*)	75
	2	22	50	2	—*)	100
	3	9	15	1	3	100
	4	18	60	2	9	100
	5	10	44	2	7	75
	Mean	15	50	1.8	6.3	90

fore, considered by us to be the cause of the results arrived at in the present investigation.

The reason why we have performed comparatively few investigations with vitamin K1 is that Stragnell (1952) has reported two deaths after the administration of 100 mg of vitamin K1 to patients with coronary thrombosis who were treated with anticoagulants; he supposed that the cause of death was fresh thromboses in the coronary vessels. In our experiments we found nothing to support this view, even though two of our patients stated that their precordial pain was aggravated after administration of

*) Patient discharged before vitamin K1 effect had subsided.

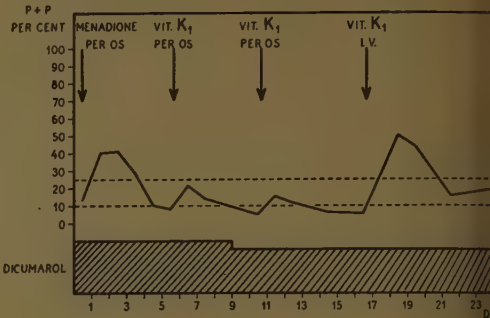

Fig. 2. Effect of menadione by mouth, vitamin K1 by mouth (two experiments), and vitamin K1 intravenously. The patient's response to vitamin K1 by mouth was strikingly slight.

vitamin K1. Like other investigators, we found that the effect of vitamin K1 was more vigorous than that of menadione. In a few cases, however, the effect was moderate. In one of these we consider that the cause has probably been defective absorption from the intestinal canal (Fig. 2).

After completing our experiments we realized that we had not administered vitamin K1 and menadione in equimolecular proportions, since 263 mg of vitamin K1 correspond to 100 mg of menadione. The use of such a high dose of vitamin K1 would probably have emphasized the superiority of this substance.

With regard to the clinical use of substances with vitamin-K effect, we consider that menadione should be used in the cases in which prothrombin concentrations lower than the therapeutic level occur in the course of treatment with anticoagulants, but in which no severe hemorrhages are present. According to our experience the therapeutic interval of the prothrombin concentration can be more safely re-established by

iistration of menadione, whilst at the time a certain, though low, maintenance of the anticoagulant is given, than by nporary discontinuance of the anti-ılant.

does not seem advisable to us to use in K_1 in the cases mentioned above. administration of this substance results often prolonged period of relative in-tiveness to anticoagulants and, as men-d above, it also involves a risk of for-on of fresh thromboses.

tamin K_1 is appropriate in the treat- of cases of overdosage with severe rrhages, and in cases where it is de- rapidly and efectively to interrupt a ment with anticoagulants. Finally, vita- K_1 may be used in intoxications with ances reducing the prothrombin con-ation, for instance the rodenticide rfarin" Holmes & Love (1952).

SUMMARY

ie effect of synthetic vitamin K and in K_1 in hypoprothrombinemia induced icoumarol, tromexan and phenylindane-e has been studied.

enadione exerts an equally vigorous ct on dicoumarol, tromexan and P.I.D., the effect is alike whether the substance ministered by mouth or intramuscularly. effect is highly varyable from one nt to another.

itamin K_1 administered by mouth also ts a uniform effect on the three anti-ulants mentioned; its effect is more vi-us than that of menadione.

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EXPERIENCES WITH A MASS SPECTROMETER IN ANALYTIC WORK WITH NITROGEN ISOTOPES

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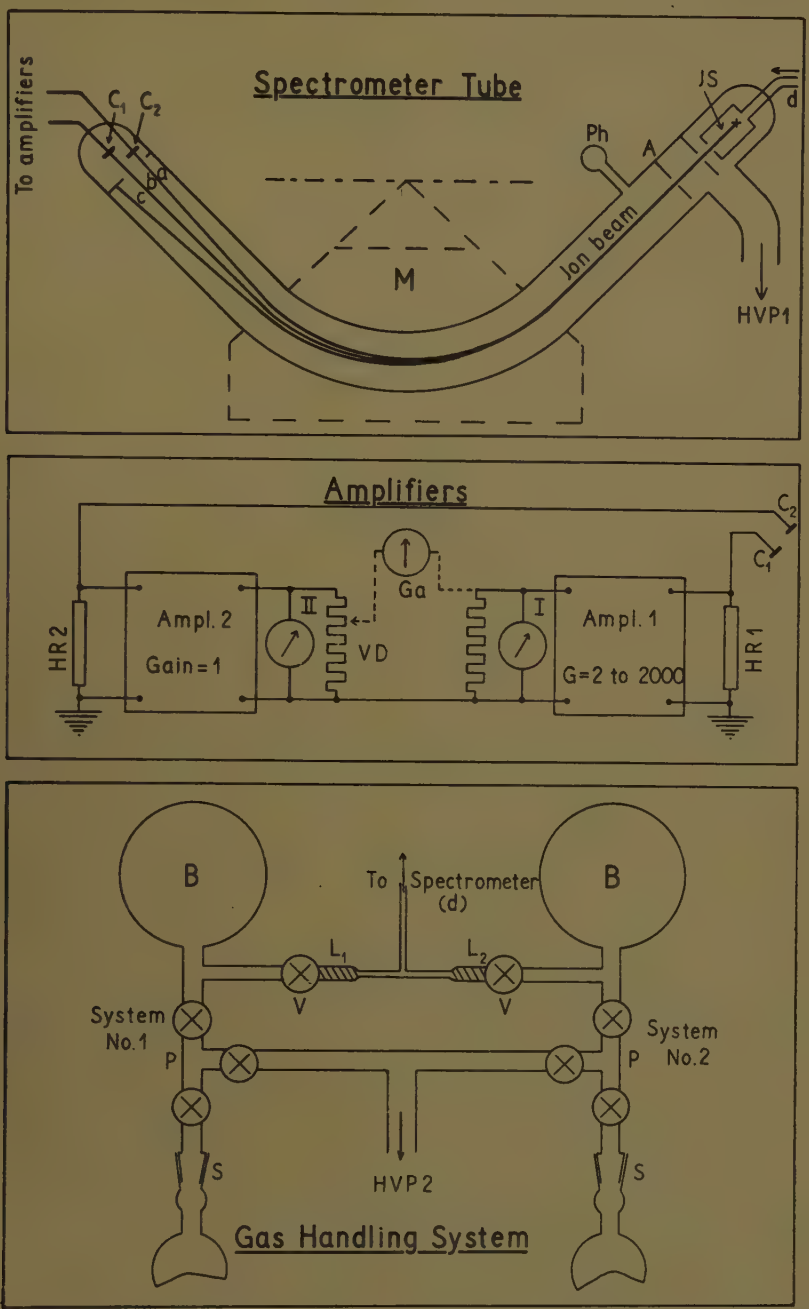
The Medical Faculty of Aarhus University has acquired a mass spectrometer, manufactured by Metropolitan Vickers, England, of Type MS 3 (J. Blears), Fig. 1. It was the first specimen of a small type instrument for analysis of lighter elements in the mass range 1 to 40, and may be used up to mass 70. In the following are presented some of the special properties of MS 3 with a few changes of the machine, together with results showing its capability for routine determinations of nitrogen isotopes.

Design of the spectrometer MS 3

A schematic diagram of MS 3 is shown in Fig. 2. After evacuation of one of the reservoirs, *B*, by means of a high vacuum pump, the gas sample to be analysed is transferred into B through the ground metal joint, *S*. By opening the membrane valve, *V*, the gas in B will flow into the spectrometer tube through a leak, *L*, of porous ceramic material, which reduces the pressure considerably. Before the gas sample is introduced, the spectrometer tube is evacuated by an oil diffusion pump with a cold trap using CO_2. The residual gas pressure is about 10^{-7} mm Hg as indicated by a Philips-manometer (Ph). In the ion source, *IS*, an electron current from a tungsten filament produces positive ions of gas present. By means of a negative potential from a rectifier giving an extremely constant DC-voltage, the accelerating voltage, which may be varied from 0 to 2000 volts, the ions are accelerated through the slit, A, and continue as a narrow beam into the tube. Across the latter is a strong magnetic field at M, in which the ion beam is deflected through 90° (radius 10.6 cm). The constant magnetic field is provided by a permanent magnet, which can be adjusted to give any desired intensity of the field.

In the left hand side of the ion tube the beam, b, passes through a slit on to the collector C_1. The current carried by the beam passes the very high resistor HR1 (20 000 Megohm). The voltage over this is amplified in the amplifier, Ampl. 1, output of Ampl. 1 is read on the meter I, deflection of which is proportional, within 0.1 cent to the intensity of the ion beam. The volt gain, *G*, in the amplifier can be varied in disc and accurately known steps, ranging from 2 to 2 Full scale on the meter I is 10 volts, correspon to 5 volts and 5 millivolts resp. over HR, depen on the range selected. On the following pages

Fig. 1. The mass spectrometer MS 3. Gas han system on the right hand front. The spectron tube is arranged behind this system. To the l the panel of the electronics.
Dimensions: 170 × 105 × 92 cm.



2. *The MS 3 spectrometer, schematically.* IS ion source, d gas inlet, A accelerating electrode, P1 pumping system, Ph Philips manometer, M magnet, C_1 and C_2 ion collectors. HR1 and 2 high ohm resistances, Ampl. 1 and 2 amplifiers, VD voltage divider, Ga balancing galvanor, I and III output meters. B reservoirs, P gas pipettes, S joints for intake of gas samples, V inlet valves, L_1 and L_2 porous leaks, HVP2 pumping system.

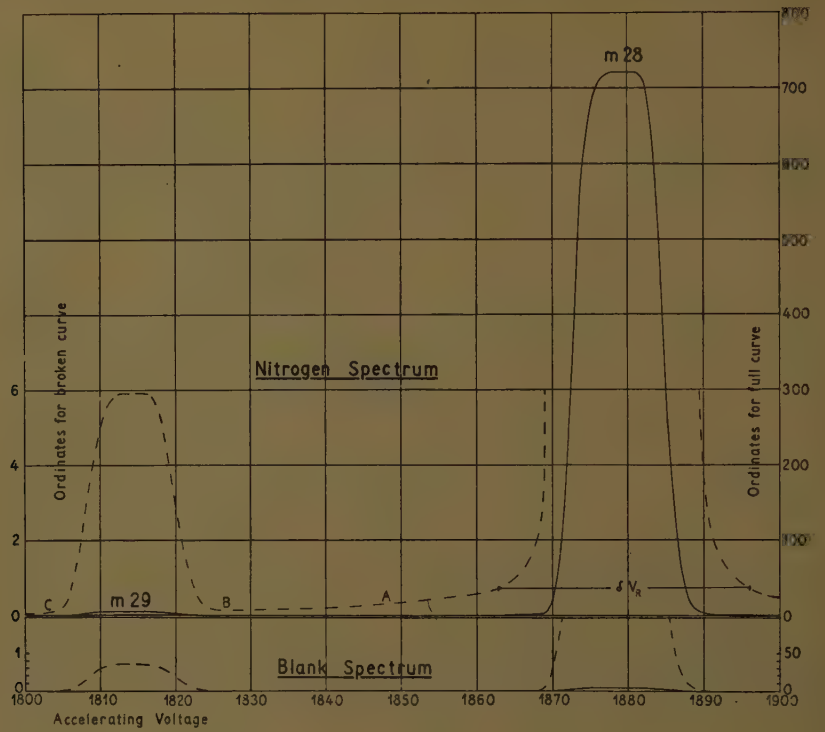


Fig. 3. *Mass spectra.* The abscissae are the accelerating voltage, while the ordinates are the re dings on the meter I (proportional to the current to collector C_1). The peak heights give the inte sities of the ion beams. The full curves correspond to the right hand scale of units. The dott curves are with ordinates in 50 fold scale (left hand scale). The upper diagram shows the ma spectrum of nitrogen gas. The lower diagram shows the "blank" spectrum of residual gases.

intensity of an ion beam is given in arbitrary units $= 2.5 \cdot 10^{-13}$ amps. corresponding to full scale of the meter at the most sensitive range.

The two vacuum systems in the MS 3 — the tube system and the gas handling system — are all-metal systems and very compact; they are evacuated by separate oil diffusion pumps. The ion tube is always baked out over the night. After the ion source has been switched on and the heating coils on the tube turned off, it takes about 1 to 2 hours before the tube is ready for exact measurements.

In the magnetic field the ion beam is split into separate beams, each containing ions with the same mass to charge ratio. They may all be measured successively on meter I.

Let the gas sample be ordinary nitrogen with the two isotopes N^{14} and N^{15}. The nitrogen ions present will be both atomic ions, $(N^{14})^+$, and $(N^{15})^+$ and molecular ions $(N^{14}N^{14})^+$, $(N^{14}N^{15})^+$, and $(N^{15}N^{15})^+$, cf. Table I. The relative masses of the molecular ions will be 28 : 29 : 30. The t corresponding beams are shown in Fig. 2, bea corresponds to m28, *b* to m29, and *c* to m30. heavier ions are deflected less than the lig in the magnetic field. When the accelerating volt is increased, the velocity of the ions rises. Ac dingly the deflection in the magnetic field decrea and the beams in the figure move downwards. reducing the accelerating voltage it is possibl make the beams *c*, *b* and *a* successively fall on lector C_1. By measuring the corresponding curr from this collector a mass spectrum is obta Fig. 3. By the *direct deflection method* are n sured the heights of the peaks, which — after rections — are equal to the amounts of ions in gas sample examined.

If a gas consists of two isotopes, as in nitro the *isotope ratio r* is defined as the numbe atoms with small mass divided by the numbe atoms with large mass. The *abundance a* is number of atoms of the large mass isotope rela

the total number of atoms, expressed in per cent. atmospheric nitrogen we have r = 273.2 and = 0.3647 atom per cent N^{15}, according to Nier 950). If the two ion beams m28 and m29 are mpared, the *ratio R m28/m29* is the number of olecules with small mass divided by the number molecules with large mass. As seen in Table I

Table I. *Isotopes in nitrogen*

		Masses	Relative amounts in ordinary nitrogen[1]	
toms	N^{14}	14	1	1
	N^{15}	15	1/273.2	1/r
	Isotope ratio:	$r = 273.2$		
	Abundance:	$a = 100/(r+1) =$		
		$= 0.3647$ per cent N^{15}.		
	$N^{14}N^{14}$	28	1	1
olecules	$N^{14}N^{15}$	29	1/136.6	2/r
	$N^{15}N^{15}$	30	1/74600	$1/r^2$
	The ratio m 28/m 29:	$R = r/2 = 136.6$.		

e have R = r/2. In ordinary nitrogen the isotope tio r is large, and accordingly the amount of 30 is very small and difficult to measure with ny precision. Very small amounts of other gases, g. NO, may contribute to the mass 30 peak. In ochemical research with slightly enriched material (a_o = 0.365) it is therefore usually preferred to easure the ratio R m28/m29, and then from R lculate the isotope ratio r (= 2R) and the abundnce a (= 100/(2R + 1)) of the isotope N^{15}. The fference between the abundances of the sample nd of the standard nitrogen gas is termed the *rcess* and may be determined with a much higher egree of accuracy than the abundances themselves ee Table III).

As seen from the mass spectrum reproduced in ig. 3 the peaks are clearly separated with only ight background. On the dotted curve (with rdinates in 50 fold scale) it is seen that this backround is less than 0.03 per cent of the intensity peak m28. The efficiency of a mass spectrometer epends on its *resolution*, which is given by the celerating voltage divided by δV_R, the width of e peak curve measured at 0.001 of the total height. Fig. 3 the resolution amounts to 90—95, or early the theoretical value of the MS3. When the solution is small the basis of a large peak will ve rise to errors due to background, when mearing neighboring peaks.

The *sensitivity* of the spectrometer is given by e intensity of m28 over the pressure in 10^{-7} mm g. The sensitivity is generally between 100 and 0, which means that the pressure in the ion tube usually will be between $10 \cdot 10^{-7}$ and $2.5 \cdot 10^{-7}$ mm Hg in analysis of nitrogen. This may be obtained with a pressure in B equal to a few tenths of 1 mm Hg.

Double collector method (D-C-method)

When it is desired to work with greatest possible precision the D-C-method must be preferred to the direct deflection method. In this method two collectors C_1 and C_2 and two independent amplifiers (Ampl. 1 and 2) are employed, cf. Fig. 2. With suitable adjustment of the acceleration voltage the beams *a* and *b*, corresponding to m28 and m29, can be focused on C_2 and C_1 respectively. By means of the amplifiers it is possible to read simultaneously the intensities of beam m28 on meter II and the intensity of beam m29 on meter I. By help of a compensating device (VD and Ga) between the two amplifiers (Fig. 2) the ratio of the two beams may be measured directly (Nier et al., 1947). The output of ampl. 1 is compensated against a known fraction (denoted *S*) of the output of ampl. 2. The galvanometer, *Ga*, serves as balance indicator. By means of a four decade precision resistance, the "voltage divider", *VD*, this balance can be set with great accuracy. From the setting S of the voltage divider, the gain of ampl. 2 (G_0=1) and the gain G of ampl. 1, the ratio R of the two beams on C_2 and C_1 is easily calculated, since R=kG/S.[1]

In the D-C-method the ratio R is thus measured directly. The simultaneous observation on both ion beams increases the precision considerably, eliminating minor changes in the ion source or in the ion tube during run.

A consequence of the high sensitivity of the instrument is that residual gases in the ion

[1] Value according to Nier (1950).

[1] Let I_1, 0_1, I_2, and 0_2 be the inputs and the outputs of the amplifiers 1 and 2 respectively, then $0_1 = I_1 \cdot G$ and $0_2 = I_2$. Further $R = I_2/I_1$ and $S = 0_1/0_2$, hence $R = G/S$. The factor *k* is added to correct for small differences in the amplifiers and the paths of the ion beams. Usually we have $k = 1.08$. A voltage divider setting $S = 0.797$ at $G = 100$ thus gives $R = 136$ or $a = 0.367$ atom per cent N^{15}.

Table II. *Intensities of masses 28, 29, and 30*

	Observed sample spectrum Pressure: $8.6 \cdot 10^{-7}$ mm Hg	Observed blank spectrum $0.6 \cdot 10^{-7}$ mm Hg	Corrected sample spectrum $8.6 \cdot 10^{-7}$ mm Hg
Mass			
m 28	790	4.30	786 ± 1
m 29	6.25	0.43	5.82 ± 0.07
m 30	0.21	0.04	0.17 ± 0.07

tube will rise to a spectrum, even when the tube is evacuated to the highest possible vacuum (about $0.5 \cdot 10^{-7}$ mm Hg). This "blank" spectrum has to be subtracted from the spectrum observed on the sample (Table II). If the subtraction of the blanks on m29 as well as on m28 is neglected, the errors on the abundance may amount to 5 and 0.5 per cent of the abundances respectively. This subtraction can be effectuated electrically by using the zero setting on the ampl. 1 to compensate the blank, before measuring an ion beam from a sample. The deflection on Meter I with the gas sample in the spectrometer will then immediately give the correct value of the intensity of the mass in question.

On the MS 3 this zero setting is on the front panel. In order to use this manner of compensating, when using the D-C-method, we had to arrange the zero setting of ampl. 2 near the front panel. On the MS 3 spectrometer this setting was originally at the rear. It is then possible, when the masses m28 and m29 are focussed on C_2 and C_1 respectively, easily to compensate either blank by means of the two zero settings. The measurements of the ratio m28/m29 with the sample in the instrument will thus give the real value of this ratio, independent of the residual gasses.

Since the "blank" spectrum may show minor changes, a further improvement was introduced in order easily to test or reset the compensating zero setting on both amplifiers during the D-C-measurements. A three position switch was built in at a convenient place on the front panel. The introduction of this switch makes it possible to carry out the above mentioned compensations of the blanks on C_2 and C_1 within a few seconds and t make an accurate determination of th abundance in such a short time (in abo 1/2 minute) that changes in the residual gas are insignificant. This improvement in th D-C-method has further increased the hig precision of the MS 3 spectrometer. It h been found of advantage to cool the io source end with a flow of air. This reduc the blank spectrum by a factor 2 to 3.

Technique of D-C-measurements

The absolute values of the ratio R and t abundance a are very difficult to find, since number of minor uncontrollable effects influenc this measurement. On the other hand, in wo with isotopes, there is often only a need for t determination of changes of the abundances. using the D-C-method alternatively on a standa gas and on samples, any changes relative to t standard gas of the abundance in the sample m be determined with high precision. This compar tive method is the basis of the technique in t following experiments.

As a standard gas we use atmospheric nitrog and a supply of air is kept in a large reservo From this a small quantity is let into system No (Fig. 2). The sample of gas to be analysed is into system No. 2. By means of the valves on t gas handling systems the pressures are regulat so that when the inlet valve ,V, from either res voir is open, the peak heights of m28 are nea equal. Thus in the spectrometer the pressure nitrogen will be of equal value for the standard a for the sample. (The two leaks were intended be equal, each giving a leak of 25 μl/sec. to atm phere, but they showed a large difference. Identi pressure in the two reservoirs resulted in pe heights with the ratio 790 to 280 or about 3 to This difference, however, had no influence on t measurements, if only the above mentioned cor tion for the pressure in the spectrometer was filled.)

The abundance in the sample is then determi as follows: 5—10 measurements on the standa

Table III. *Determination of excess in abundance*

Comparison of sample with the standard gas or the ratio m28/m29.
Voltage divider settings:

Standard before S_o	Sample S	Standard after S_o
0.7978	0.8100	0.7975
0.7971	0.8110	0.7980
0.7975	0.8119	0.7979
0.7971	0.8115	0.7980
0.7974	0.8113	0.7975
	0.8117	
	0.8116	
	0.8117	
	0.8116	
	0.8119	

Mean of standard: $S_o = 0.7976 \pm 0.0001$; Gain $G_o = 100$.
Mean of sample: $S = 0.8114 \pm 0.0002$; Gain $G = 100$.

From the standard values of S_o and G_o the value of the constant k is calculated by means of $R_o = k\ G_o/S_o$, when it is assumed that $R_o = 136.60$ for the standard gas (atmospheric nitrogen). The ratio R in the sample is thus $R = 136.60\ (S_o G)/(S\ G_o)$. We find:

$$R = 134.28 \pm 0.02$$

From R is calculated the abundance
in the sample: a = 0.37098 atom per cent N^{15}.
in the standard: $a_o = 0.36470$ » » »
Excess in the sample:

$$a\text{-}a_o = 0.00628 \quad » \quad » \quad »$$

with an error of $\pm$ 0.00006 atom per cent N^{15}.

Table IV. *Repeated measurements of the abundance* a *in a gas sample, prepared from commercial* NH_4NO_3

0.36364	atom	per	cent N^{15}
0.36335	»	»	»
0.36350	»	»	»
0.36345	»	»	»
0.36353	»	»	»
0.36358	»	»	»
0.36348	»	»	»
0.36345	»	»	»
0.36366	»	»	»
0.36366	»	»	»

Mean: a = 0.36353 atom per cent N^{15}.
Error on the mean: $\pm$ 0.00004 or $\pm$ 0.01 per cent of the abundance value.
Error on the single observation: $\pm$ 0.00013 or $\pm$ 0.04 per cent of the abundance value.
Excess relative to atmospheric nitrogen ($a_o = 0.36470$):

$$a\text{-}a_o = -0.00117 \text{ atom per cent } N^{15}.$$

Table V. *Measurements of the abundance* a *on different gas samples, prepared from a specimen of slightly enriched* NH_4NO_3

0.3968	atom	per	cent N^{15}
0.3970	»	»	»
0.3959	»	»	»
0.3952	»	»	»
0.3963	»	»	»
0.3971	»	»	»
0.3974	»	»	»
0.3974	»	»	»
0.3971	»	»	»
0.3963	»	»	»
0.3959	»	»	»

Mean: a = 0.3966 atom per cent N^{15}.
Error on the mean: $\pm$ 0.0002 or $\pm$ 0.05 per cent of the abundance value.
Error on the single observation: $\pm$ 0.0007 or $\pm$ 0.18 per cent of the abundance value.
Excess relative to atmospheric nitrogen ($a_o = 0.36470$):

$$\triangle a = a - a_o = +0.0319 \text{ atom per cent } N^{15}.$$

hen 10 measurements on the sample and again 5—0 measurements on the standard. Each observation akes about ½ minute. Table III gives an example f the observations. We see from these typical neasurements that for an isotope which, as N^{15}, s present only to an extent of about 0.4 atom per ent of the total nitrogen, it is possible to detect ifferences in the abundances of less than $\pm$ 0.02 er cent of the value of the abundance.

The reproducibility of the measurements with MS 3 is also seen from series as shown in Tables V and V. The first represents the results of epeated determinations of the abundance on the ame gas sample, while Table V gives the results f observations on 11 gas samples, prepared from he same solution of nitrate of ammonia. The error on the mean is in the first case 0.01 per cent and 0.05 per cent in the latter case, where error due to the chemical procedure will be included in the results. It must however be added that it is only possible to obtain this high accuracy, when the abundance of the standard and the sample are of the same order of magnitude.

It is a further condition for obtaining a great precision that a correction is introduced for the

Table VI. *Change in abundance during run to the spectrometer tube. Reservoir 1 litre.*

Standard gas: Air, $a_o = 0.36470$ atom per cent N^{15}. Gas: Air.

Time min.	Peak height m28 units	Abundance a	Change in abundance $a - a_o = \Delta a$
0	906	0.36470	0
55	848	0.36518	0.00048 (0.13 per cent)
95	793	0.36567	0.00097 (0.26 » »
135	752	0.36605	0.00135 (0.37 » »

Change in 10 min.: $\Delta a = 0.03$ per cent.

admixture of air to the samples for analysis. In the preparation of nitrogen gas from biological material the authors have carefully followed the advice given by Sprinson & Rittenberg (1949), where oxidation of ammonia to nitrogen is effected by hypobromite in glass ampullae. In Fig. 1 one of the glass ampullae used (manufactured by Nordisk Glasteknik, Copenhagen) is fastened to the system on the right hand side. Air is indicated by the occurrence of an argon peak. The ratio of nitrogen to argon, and thereby our correction factor for the admixture of air to the samples for analysis, has changed during the work. It has usually been about 60 to 80. In the majority of the samples for analysis the admixture of atmospheric air has been about ¾ per cent.

Experience with the gas handling system

Pressure. With increasing pressure in the reservoir the peak height increases. With the leaks used, which were rather wide, we did not, however, find proportionality. In the spectrometer tube itself approximate proportionality is found between peak heights and pressure as measured on the Philips manometer.

Rate of pumping out. When series of measurements are made, it is of importance that the reservoirs of the gas handling system are completely and rapidly evacuated. This may be controlled by observing the reduction of the peak height during the evacuation. Thus the peak height was reduced from about 800 to 1/1000 and 1/10 000 in 90 sec. and 120 sec. respectively, when the reservoir was 1 litre. The evacuation at pressures below 0.1 mm Hg followed strictly the laws of diffusion. The reservoir is ready for a new sample within 2 minutes. If the new sample has an abundance which is very di ferent from the first sample, a memory effe is observed decreasing with time. The m mory effect when going from a highly to less enriched sample is indicated by a co siderable increase in the voltage divid reading for the standard air. This increasi effect is negligible for samples with an e cess below about 0.3 per cent. Samples wi excesses between 0.3 and 1 per cent w produce a memory effect for approximate 20 minutes, and with greater excesses it m be necessary to wait several hours before memory effect has disappeared.

Fractionation. It is important to kn whether fractionation of a gas sample curs, when handling the gas in the reservoi

A. When the gas is taken from the servoir into the spectrometer through leak, this will be a diffusion process, a accordingly the abundance in the reserv will increase. To see how far this was case, an experiment was made in which change in abundance was followed for se ral hours, while the gas was streaming c tinually into the spectrometer. In Table the abundance is given relative to standa and it is seen that it increases proportiona to time. The increase, however, is so sl that in the 10 minutes which usually suff for a series of measurements, the error abundance will be negligible when using litre reservoir.

From the data on the decrease in p height and the volume of the reserv (1 litre), the amount of gas used may

able VII. *Comparison of the abundance values, as measured from reservoir 1 through leak 1 and from reservoir 2 through leak 2*

Gas: Atmospheric air. Abundance $a_o = 0.3647$ atom per cent N^{15}. The pressure in the reservoir 2 is regulated by pumping out to give different peak heights for m28.

Reservoir 1 Peak height units	Reservoir 2 Peak height units	Difference in abundance $a - a_o = \Delta a$
900	900	0
900	600	0.0025 (= 0.65 per cent)
900	300	0.0050 (= 1.30 per cent)

imated. In 10 minutes only 5—10 μl rogen gas (at NPT) or 0.25—0.5 micro-ls stream into the spectrometer. The mini-um amount of gas required for an analysis ll be at least 100 times larger in order to oid too large changes in pressure during observation.

B. Under certain conditions a fractiona-n also takes place during the evacuation the reservoirs by means of the gas hand-g diffusion pump, cf. Table VII. In both ervoirs air was introduced giving the ne peak height for m28. When reducing pressure in res. 2 by help of the diffusion mp, an increase in the abundance of the avier isotope is clearly shown as compared th the abundance of the air in res. 1. If base effect, described later, is considered, gher. When comparing gas samples in the o reservoirs one should always adjust the essure to give the same peak heights for Δa values in Table VII will be still 8. If it is necessary to reduce the pressure one reservoir, reducing by expansion must preferred to pumping out.[1]

[1] The changes in the observed abundances, men-ned at A and B have now also been established experiments with new leaks of equal size, and h pure nitrogen gas. These effects, however, re only observed on the gas inlet system from 2 and not from res. 1, and an interchange of two porous leaks did not alter this fact. The hors are not able to give an explanation of this erent behavior of the two gas inlet systems. obably there is an unintended asymmetry of the systems. (Added in proof.)

Error from neighboring peaks

If a small peak, e. g. m29, is close to a high peak, e. g. m28, then the base of the high peak may give rise to errors. In Fig. 3 it will be seen that the base of the peak m28 extends below peak m29. From the slope of the base (ABC in the figure) the contribution β of this base to the total peak m29 may be estimated. Since it can be shown that the increase in β is not proportional to the peak heights h, but approximately proportional to $h^{1.6}$, it follows that the presence of the base β will change the observed abundance values, when the peak height is changed by altering the pressure. From the values of β it can be calculated that if the peak m28 in ordinary nitrogen is reduced from e. g. 800 units to 400 units, the observed abundance should diminish from a to (a—0.0020). This was confirmed by a series of measurements in which the pressure in the reservoir was reduced by expansion, i. e. without fractionation. Although the actual abundance was unchanged, the observed abundance as measured by the D-C-method was diminished at the same rate as calculated from the base effect. Thus the base effect must be considered, when abundances observed at unequal peak heights are compared. In the previously described D-C-method relative to a standard, this claim of equal peak heights of m28 is considered, and accordingly the base effect is eliminated, at least when the difference between the abundances in the standard gas and in the sample is not too large.

Table VIII. *Analysis of known material*

NH_4NO_3-solutions enriched with N^{15} in the NH_4-group.

Solution	Calculated atom per cent N^{15}	Observed atom per cent N^{15}
1	9.707	9.715
2	5.060	5.057
3	2.9171	2.9168
4	1.5583	1.5600
5	0.7501	0.7521
6	0.3904	0.3905

Accuracy on actual observations

From a preparation with enriched nitrogen, NH_4NO_3, with 36 atom per cent N^{15} in the ammonia group, a series of gas samples were made after dilution of the enriched material with ordinary NH_4NO_3. From the amounts of the components the abundances of the samples were calculated. On this known material the abundances were measured in the mass spectrometer (Table VIII). The calculated and the observed abundances agree within the experimental error, which was less than 0.1 per cent. The discussion of a series like this involves a number of corrections, which we shall omit here, but we have given the table to show the high precision which may be obtained with the MS 3-machine.

In biochemical research, during which a large number of mass spectrometrical analyses were done, the mean error on the results of 4-fold determinations of samples with varying abundances were calculated to be about 0.1 per cent of the abundance values.

One of the difficulties encountered in precision work on nitrogen isotopes is that the absolute value of the abundance in the standard gas, e. g. ordinary nitrogen, is known. In the literature are found the val given in Table IX. In this paper we h adopted the value by Nier as being the b Since, however, our machine is capable giving the fourth and even the fifth deci in the abundance we have to assume a fi value of $a = 0.36470$ for our calculati and the error in this standard value will included in the results. The determination an excess in abundance is independent this error, since the excess is the differenc two (nearly equal) abundances.

It will be possible, from a series measurements like those given in Table to redetermine the standard value of abundance for nitrogen. As a prelimin result of such a series it was found $a = 0.370 \pm 0.004$ (corresponding to 269.4 and $R = 134.7 \pm 1.4$). This valu markedly higher than the value given Nier (1950).

SUMMARY

It has been outside the scope of this pa to give a general review of mass spect metry. On the contrary we have wished present the results of some of the exami tions which must be performed when st ing work with mass spectrometry by help a new type of mass spectrometer.

The results presented indicate that MS 3 spectrometer is a very efficient chine for routine analyses of nitrogen topes. The determinations of these isot can be carried out in short time and great precision. In connection with double collector method, some minor alt tions of the apparatus are described wh have increased the accuracy of the meas ments considerably.

Table IX. *Absolute abundance of atmospheric nitrogen*

r	R	a		
274.4	137.2	0.3632	Urey et al.	1931
265 ± 8	133 ± 4	0.376 ± 0.011	Vaughan et al.	1934
265	132.5	0.376	Rittenberg	1946
273.2 ± 0.8	136.6 ± 0.4	0.3647 ± 0.0011	Nier	1950

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THE PREPARATION OF PROTHROMBIN-FREE OX PLASMA IN THE ESTIMATION OF PROTHROMBIN

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During the last few years Owren's method (1949) for the determination of the combined effect of prothrombin and proconvertin has been introduced in clinical laboratories as one of the most exact and reliable methods for the estimation of prothrombin activity in blood. Several investigations have demonstrated the practical (Owren 1951, T. Astrup, Müllertz & Rud Hansen 1951, Bjerkelund 1953) and the theoretical (Müllertz 1953) advantages of this method.

The prothrombin-free ox plasma used in Owren's method is prepared by filtration through asbestos filter pads and complies with the following requirements: it is free from prothrombin and proconvertin; it contains sufficient amounts of proaccelerin as well as of fibrinogen for the coagulation process. Such plasma has proved to give consistent results. We have tried by treatment of ox plasma with barium sulphate to produce an absorbed plasma presenting the same qualifications.

500 ml ox plasma obtained from oxalated blood (100 ml 2.5 per cent potassium oxalate solution per liter blood) was stirred mechanically at room temperature with 10 g of barium sulphate (Pharmacopea Danica 1948). Samples were removed after 30 min. (B), 40 min. (C), 50 min. (D) and 60 min. (E) and centrifuged for 10 minutes (2500 rpm). The supernatants were collected and stored at —20° C.

With this $BaSO_4$ treated plasma, dilution curves of prothrombin-containing blood samples were determined in the usual way (Owren 1949).

Normal oxalated human plasma was used in stock dilution 1:10. Serial dilutions of this stock dilution were prepared and 0.2 ml of each were mixed with 0.2 ml thromboplastin and 0.2 ml ox plasma. Finally 0.2 ml of a calcium chloride so tion (0.030 mol.) were added and the clotting ti measured. The logarithms of the clotting tim were plotted on the ordinate against the logarith of the prothrombin concentrations.

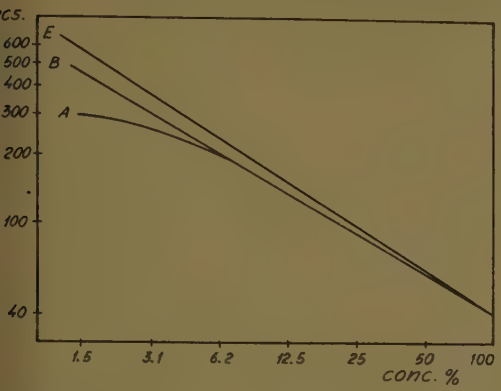
In Fig. 1 some of the curves are presented. $BaSO_4$ samples yielded curves with steeper slo than those obtained with asbestos filtered ox plas (A), the divergence being most marked for longest clotting times. This indicates that samples treated with $BaSO_4$ are completely f from prothrombin, whereas the asbestos filtered plasma still contains small amounts of prothromb All samples treated with $BaSO_4$ can presumal be used, however we recommend that plasma stirred for 30 or 40 minutes. The samples w stable for more than 6 months when stored —20° C. Identical results were obtained with ferent batches of ox plasma.

Smaller abounts of $BaSO_4$ and shorter stirr times were also tried. Such samples yielded cur which deflected downwards from the straight at low prothrombin concentrations, indicating presence of a trace of prothrombin similar to that the filtered ox plasma.

Larger amounts of $BaSO_4$ were found to int fere with other components of the plasma yielded inferior products.

For years it has been known that p thrombin can be removed from ox plas by means of different adsorbants such calcium triphosphate, aluminum hydroxi calcium fluoride, barium carbonate a barium sulphate. Barium sulphate see preferable because it is insoluble, neut and is available in constant quality with p ticles not greater than 1 μ, according to description in Pharmacopea Danica.

$BaSO_4$ has been used to remove p thrombin by Bordet & Delange (19 and later by Frommeyer (1949), Jaq



Legends for Fig. 1: Prothrombin dilution curves lotted in a double logaritmic graph.
Abscissa: Relative prothrombin concentration.
Ordinate: Clotting time.
A: Asbestos filtered ox plasma.
B: 2 g barium sulphate per 100 ml plasma. 30 min.
E: As in B, but stirred for 60 min.
(Individual readings were omitted for typographical reasons).

1943), Owren (1947) and Surgenor (1952). lasma treated with large amounts of $BaSO_4$ ere used in the estimation of prothrombin plasma by Tanturi (1946).

The concentrations of proaccelerin in nor- al human plasma are just sufficient to give a maximal acceleration of the coagulation process. Ox plasma has a content of proaccelerin twice or three times as great as human plasma, Owren (1947), Ware (1947). Owren found that not more than 50 per cent of the proaccelerin was removed by treatment with $BaSO_4$. Accordingly $BaSO_4$ treated ox plasma still contains an excess of proaccelerin.

$BaSO_4$ treatment of fibrinogen does not alter the nitrogen content of the sample (Naninga 1947) and produces no structural changes of the fibrinogen (Fantl 1948, Gollub 1949).

The ox plasma as here prepared is suitable for the estimation of prothrombin concentrations and presents the following advantages: The preparation is simple, rapid and needs only inexpensive equipment.

SUMMARY

Oxalated ox plasma treated with barium sulphate (2 g) per 100 ml) is suitable in the estimation of prothrombin activity by the method described by Owren. The preparation is simple and cheaper than the original procedure.

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FAILURE OF CATION EXCHANGE RESIN TO IMPAIR INTESTINAL ABSORPTION OF TERRAMYCIN

On the basis of their experiments *in vitro* Friedman et al. (1953) reported that antibiotic substances which can be absorbed by cation exchange resins may lose their antibacterial activity, if they first come in contact with the resins. Such antibiotics were dihydrostreptomycin, terramycin, and aureomycin. If for instance terramycin and aureomycin were incubated for five hours together with cation exchange resin, they lost their antibacterial quolities. Against gram negative organisms they became inactive immediately after the incubation. But the cation exchange resins had no inactivating influence upon penicillin and chloramphenicol.

The object of our investigation has been to ascertain whether cation exchange resin given at the same time as terramycin will influence upon the intestinal absorption of the latter. For that purpose we first gave six hospital patients for one day only terramycin, dose: 0.5 g every sixth hour. After that the terramycin dosage continued in the same way, but in addition to that was given 10.0 g cation exchange resin (»Carboresin«) three times a day for two days. On the fourth day again only terramycin was given.

During the trial blood samples were taken three times daily and the terramycin levels of serum were estimated (Fig. 1).

METHODS

The active terramycin content in the serum v ascertained as follows: The sera were titrated twofold dilutions in semifluid agar, to which v added one drop of a 4-hour broth culture Staphylococcus aureus P 209 diluted to 1 : 200. T sensitivity of the strain to terramycin has remai rather stable at a value of 0.15 microg./ml.

The reading of the results was performed af an incubation of 24 hours by noting the maxim dilution producing total inhibition. *In all ca the control sample of serum taken before the ter mycin treatment showed no inhibition.*

As can be seen in figure 1 there were changes in the terramycin level of the ser during the cation exchange therapy. Th we have not been ably by our clinical expe

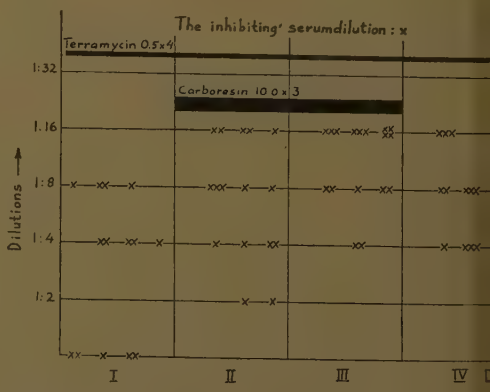

Fig. 1. Terramycin levels of serum during Ter mycin and cation exchange resin therapy in six cases.

ents to confirm the above mentioned in-stigators' test results in vitro in respect to rramycin.

SUMMARY

The authors have examined the influence clinical doses of cation exchange resin on the intestinal absorption of terramycin hen given at the same time as the latter. It was not observed to have any influence upon the terramycin levels of the sera of six examined patients.

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DETECTION OF SOME NEW ABNORMAL METABOLITES IN THE URINE OF PHENYLKETONURIA

Previous work on phenylalanine metabolism in phenylketonuria has been reviewed recently by Berg (1953) and Lerner (1953). The metabolic error has been ascribed by Jervis (1947) to the inability to hydroxylate phenylalanine in the para position. This concept would explain the known pathological excretion in the urine of phenylalanine, phenylpyruvic, phenyllactic and phenylacetic acid.

The application of paper chromatography (Boscott 1952 a, b, c) to the study of the acidic and phenolic fractions I, II, III (Boscott 1952 a) of the urines from 15 patients with phenylketonuria has now revealed further abnormal phenolic compounds, the nature of which suggests hitherto unrecognised metabolic disturbances in phenylketonuria.

Three of the patients were children whose urine was collecting while receiving (a) a normal diet, (b) the same diet with daily supplements of from 1—10 grams dl-phenylalanine, and (c) a diet free from phenylalanine (Bickel, Gerrard & Hickmans 1953).

Fig. 1 with descriptive key illustrates the total number of phenolic acids that have been found in Fraction I of the urines from 160 normal and hospitalised individuals. Every chromatogram was investigated by the following tests of detection and in the order given:

1. Fluorescence of the chromatogram at neutral pH in U. V. light (Wood's glass filter).
2. Fluorescence with microdrops of 90 per cent v/v H_2SO_4 spaced at intervals of about 2 cm.
3. Fluorescence and colour changes after sprayi the paper with 10 per cent Na_2CO_3 soluti
4. Formation of azo dyes by spraying with diaz tised p-aminophenyldiethylaminoethylsulpho (ICI 5091).
5. Colour changes after spraying with 2N KO shortly after 4.

The chromatographic pattern of the ph nolic acids in Fraction I was practical identical in the urine of all phenylketonur patients but showed some striking deviatio from the normal pattern. Phenylpyruv acid was readily detectable while the patien received diets (a) and (b) but disappear on the phenylalanine-free diet (c). Relative large amounts of compound 29 (p-hydrox phenylacetic acid) and lesser amounts compound 26 (p-hydroxyphenyllacetic aci were found consistently in all phenylketo uric urines; these substances have previous been recorded in the urine of scorbu guinea pigs (Aterman, Boscott and Coo 1953). The methods employed do not perm the detection of p-hydroxyphenylpyruv acid though this compound has recently be demonstrated by another method in t urine of patients with phenylketonuria (Re 1950). It is also known to be excreted scorbutic individuals.

Compound 30, identified tentatively o-hydroxyphenylacetic acid, was excreted abnormally high amounts in the urine phenylketonuric individuals whereas co pound 28 (m-hydroxyphenylacetic ac which was invariably found in the cont urines was not excreted by these patien

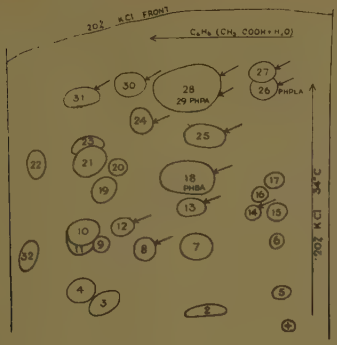

Fig. 1. *Map of the spots of Fraction I* (only the phenolic acids are shown).

Key. Colour of dyes (Na_2CO_3):

Compounds	5, 6, 17 —	yellow
—	15, 16, 25 —	orange-yellow
—	2, 7, 18, 19, 22, 24, 27, 28 —	orange
—	8 —	orange-pink
—	30 —	salmon-orange.
—	12, 14, 20 —	pink.
—	3, 10, 21, 26, 29, 32 —	red.
—	9 —	magenta.
—	11, 23, 31 —	violet.
—	4, 13 —	mauve

Phenylpyruvic acid (revealed by test 5 between 22 and 31) — yellow dye — bluish halo.

Compounds indicated with arrows found in urine of normal rat fed purified diet. Other compounds are derived from dietary phenols other than tyrosine.

Method: Capillary ascent with Whatman paper No. 542 (26.5 × 37.5 cm). Amount of Fraction I used was equivalent to $\frac{1}{2000} - \frac{1}{200}$ of a 24 hr. specimen urine. 1st solvent benzene-acetic acid — water 2:2:1; 2nd solvent 20 per cent KCl 34° C (no organic solvent present).

Fractions II and III of phenylketonuric urine revealed consistently the presence of large amounts of 5-benzalhydantoin, an artefact easily formed from urea and phenylpyruvic acid. The excretion of this compound as well as of compound 30 was greatly elevated when additional phenylalanine (diet b) was fed, and reduced on the phenylalanine-free diet (c). The excretion of compounds 26 (p-hydroxyphenyllactic acid) and 29 (p-hydroxyphenylacetic acid) was almost unchanged after phenylalanine addition (diet b) since their presence in phenylketonuric urine seems to depend only on the tyrosine intake.

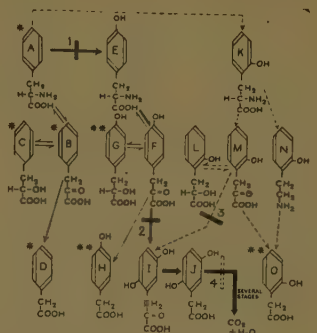

Fig. 2. Metabolic pathway in phenylketonuria. Key: * Previously known abnormal metabolites, ** newly recognised abnormal metabolites. ➡ Normal metabolic pathway, → established pathway in phenylketonuria, ⇢ hypothetical pathway in phenylketonuria.

Fig. 2 summarises our somewhat hypothetical views on the phenylalanine metabolism in phenylketonuria. It will be seen that block 2 simulates block 1 in that the conversion of compound F to I also requires the introduction of a hydroxylic group para to a substituent in the benzene ring. The conversion of A to K, which must be relatively insignificant in normals, may well increase in phenylketonurics, and will be followed by subsequent degradation to N (compound 30). Block 2 rather than block 4 would appear to be responsible for the incomplete metabolic breakdown of the benzene ring of phenylalanine or tyrosine in phenylketonuria (c. f. Penrose & Quastel 1937) since no homogentisic acid was found in the urines examined. The results obtained with diets (a) and (b) strongly suggest that B, C, and D are not converted by direct para hydroxylation to F, G and H. The accumulation of A but not of abnormal amounts of E or K in the blood and urine of phenylketonuric patients may be due to the faster metabolic degradation of compounds E and K because of the activating influence of a hydroxyl group.

The pathological significance of the newly detected phenolic compounds is not yet clear. It is possible that the tyrosine analogue o-hydroxyphenylalanine and its metabolites L, M, N and O represent — in the presence of a metabolic block between phenylalanine and tyrosine — an alternative metabolic pathway of phenylalanine degradation, similar to the established pathway through phenylpyruvic, phenyllactic and phenylacetic acid. The increase of the para hydroxy compounds can only be understood if a further metabolic block in the breakdown of tyrosine metabolites is assumed, but this second block may be closely related to the first, if one assumes that one enzyme whose function it is to hydroxylate the compound in question is missing. How far the newly detected compounds rather than phenylalanine, phenylpyruvic, phenylactic or phenylacetic acid are responsible for the mental changes in phenylketonuria is still a matter of conjecture, but this question may be clarified by feeding experiments with such substances.

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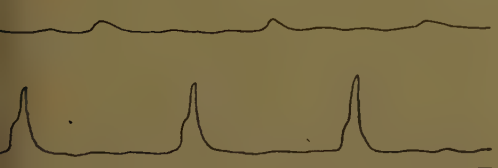
PRESSURE RECORDINGS IN THE UPPER URINARY TRACT

Most of the information concerning the ffect of drugs on ureteral activity is based n the recordings obtained with Trattner's reterometric apparatus and later modifica-ons.

Morales *et al.* (1952) in experiments on ogs found that these kymographic records ive an erroneous picture of increased peri-altic frequency with diuresis and concluded iat the preferable way of recording the requency of ureteral contractions was by irect observation.

Physically adequate methods of recording hasic pressure changes are in extensive use specially in cardiovascular research but ave, to my knowledge, not previously been pplied to studies of the function of the upper rinary tract.

At Department III, Ullevål Hospital, I ave, with the support of Professor C. Semb, ad the opportunity to study these pressures n connection with routine diagnostic cysto-copies.



Pressure curves from upper part and middle part of ureter.

Using two Statham physiological pressure transducers, recordings can be obtained simultaneously from both ureters, from the bladder and one ureter or from two levels in the same upper urinary tract.

To allow for the use of ordinary plastic ureter catheters, a simple connecting unit was constructed.

The pressure transducers are connected to a two channel oscillator-amplifier. Permanent chart records are obtained with a Brush Oscillograph, which was modified to give chart speeds down to 1 mm/sec. The catheters are filled with urine.

The question then arises whether retention will occur proximal to the tip of the catheter when the urine is not allowed to flow through it.

In the presence of a ureteral obstruction, a pressure increase would then soon develop due to the small volume of the renal pelvis and the upper part of the ureter and the resistance to the outflow would increase, the further the catheter is introduced. The highest pressures would then be found proximally in the ureter.

In the figure the upper curve is obtained at a level of 25 cm from the ureter orifice, the lower curve is recorded immediately afterwards, at a level of 10 cm.

It is shown that the pressure after each period of activity decreases to the former value, the resting pressure.

It is further evident that the resting pressure in the upper part of the ureter is the same as in the middle part of the same

ureter and that the pressure amplitudes are much lower.

The resting pressures remain on the same level even when the recordings are continued for more than one hour.

By using this method it is possible to study how the urine portions are transported from the calyces to the bladder, how drugs and changes in diuresis affect the ureter activity and how the pressures are chang during different diseases of the upper urina tract.

A more detailed description of the meth and presentation of the results will appe later.

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Nov. 11, 1953. *F. Kiil.*

INDEX OF AUTHORS

(L) = Letters to the Editors, (T) = Technique and Practical Problems

rvidson, M., Hedlund, P. & Mossberg, H.-O.: An agglutinogen skin test in pertussis 44
strup, T.: The toxicity of synthetic heparin substitutes 137
terman, K., Boscott, R. J. & Cooke, W. T.: Acidic and phenolic compounds in hydrolysed human urine, with particular reference to disorders of the liver 296 (L)

ickel, H.: see *Boscott, R. J.* 380 (L)
rd, G. W. G.: The significance of auto-agglutinins 143
legen, E. M.: see *Haugen, H. N.* 58
legen, E. M.: see *Haugen, H. N.* 63
legen, E. M.: see *Haugen, H. N.* 67
oscott, R. J. & Bickel, H.: Detection of some new abnormal metabolites in the urine of phenylketonuria 380 (L)
oscott, R. J.: see *Aterman, K.* 296 (L)
rendstrup, P.: On the unsaturated copper-binding capacity of blood serum 18
rendstrup, P.: Serum iron, total ironbinding capacity of serum, and serum copper in normals 312
roch, O. J.: The base-binding power of serum proteins and their function as osmoregulators in body fluids 9
rodthagen, H.: The preparation of prothrombin-free ox plasma in the estimation of prothrombin 376 (T)
rohult, A.: β-globulin concentration and treatment of infantile leukemia 328

ooke, W. T.: see *Aterman, K.* 296 (L)

edichen, J.: Long term anticoagulant treatment of cardiovascular disorders with phenylindandione (PID-thrombantine) 324
encker, J. S.: Studies in artificial peripheral embolism in the rabbit 261
evey, J. D.: An improved reagent for oxygen absorption 104 (L)
iamant, H. & Tammelin, L.-E.: An electrometric method for the determination of cholinesterase activity II 271

Dragsted, P. J. & Hjorth, N.: Urinary calcium output during treatment with ACTH 188
Ekman, B., Thune, S. & Truedsson, E.: Investigation of viscosity and hyaluronidase inhibitors in joint exudate after intraarticular cortisone injections 175
Engbæk, H. Chr.: Precautions against infection of personnel working in tbc. laboratories 198 (T)
Eriksen, L.: Paper chromatography of porphyrin pigments 155
Euler, U. S. von, Lund, A., Olsson, A. & Sandblom, Ph.: Noradrenaline and adrenaline in blood and urine in a case of phaeochromocytoma 122

Felkai, B.: see *Halmágyi, D.* 85
Foss, O. P.: On the determination of glutamine and glutamic acid in urine by a modification of Krebs' method 334

Goa, J.: A micro biuret method for protein determination 218
Grette, K.: Mircomethod for the determination of calcium and magnesium in serum 151
Grüner, A., Hilden, K. & Hilden, T.: The effect of heparin and protamine sulphate on the occurrence of chylomicrons in human blood 241
Grüner, A. & Hilden, T.: The occurrence of chylomicrons in the blood in young and old individuals 236
Grönroos, J.-A. & Kalliomäki, L.: Failure of cation exchange resin to impair intestinal absorption of terramycin 378 (L)
Hallman, N.: see *Leppänen, V.* 250
Halmágyi, D., Iványi, J., Felkai, B., Zsóter, T., Tényi, M. & Szücs, Zs.: The effect of dihydroergotamine and hydergin on pulmonary arterial pressure in man 85
Haugen, H. N.: The determination of endogenous creatinine in plasma and urine 48
Haugen, H. N. & Blegen, E. M.: The renal response to hexamethonium 58
Haugen, H. N. & Blegen, E. M.: Blood urea and plasma endogenous creatinine in renal insufficiency 63

Haugen, H. N. & *Blegen, E. M.:* The true endogenous creatinine clearance 67
Hedlund, P. & *Lagerkrantz, R.:* Typing of hemolytic streptococci in scarlet fever and other streptococcal diseases 39
Hedlund, P.: see *Arvidson, M.* 44
Heilbronn, E.: An electrometric method for the determination of cholinesterase activity III 308
Heilskov, N. S. C., Olesen, K., Schönheyder, F. & *Werner, S.:* Experiences with a mass spectrometer in analytical work with nitrogen isotopes 366 (T)
Hilden, K.: see *Grüner, A.* 241
Hilden, T. & *Munck, O.:* Vitamin K as antagonist to anticoagulants 361
Hilden, T.: see *Grüner, A.* 236
Hilden, T.: see *Grüner, A.* 241
Hjort, P.: The effect of an anion exchange resin on pepsin 350
Hjorth, N.: see *Dragsted, P. J.* 188

Iványi, J.: see *Halmágyi, D.* 85

Jacobsson, K.: Electrophoretic demonstration of two trypsin inhibitors in human blood serum 97 (L)

Kalliomäki, L.: see *Grönroos, J.-A.* 378 (L)
Kamer, J. H. van de: Quantitative determination of the saturated and unsaturated higher fatty acids in fecal fat 30
Kennedy, G. Y.: A new apparatus for column chromatography 201 (L)
Kennedy, G. Y.: Partition chromatography of the porphyrins 281
Kiil, F.: Pressure recordings in the upper urinary tract 383 (L)
Krook, H.: Estimation of portal venous pressure by occlusive hepatic vein catheterization 285
Kulonen, E.: Paper partition chromatography in the study of keto acids II 72
Köiw, E.: A photometric scanning device for paper electrophoresis 99 (L)

Lagerkrantz, R.: see *Hedlund, P.* 39
Larmi, T. K. I.: Variations in the oxygen and carbon dioxide tensions of end-tidal air in graduated exercise in normal persons, and patients suffering from ventilatory insufficiency 90
Laurell, C.-B.: The diurnal variation of serum iron concentration 118
Laurent, B. & *Nikkilä, E. A.:* Studies on experimental proteinuria I 158
Lehmann, J.: A photoelectric micro method for the direct titration of calcium in serum with ethylene-diamine tetracetat 203 (L)
Leppänen, V. & *Hallman, N.:* Improved instrument for internal standard flame photometry 250
Lund, A.: see *Euler, U. S. von* 122
Lundin, J.: An arrangement for cleaning pipet 293 (T)
Malm, O. J.: On phosphates and phosphoric a as dietary factors in the calcium balance man 75
Milthers, K. & *Roholdt, K.:* Inactivation of benz penicillin-β-diethylaminoethylester hydriodide the blood 102 (L)
Mossberg, H.-O.: see *Arvidson, M.* 44
Munck, O.: see *Hilden, T.* 361.
Müllertz, S.: Dilution curves and accuracy proatrombin estimations during hepatitis dicumarol therapy 179

Nieweg, H. O.: The effect of liver extract in c tures of megaloblastic bone marrow 195
Nikkilä, E. A.: see *Laurent, B.* 158

Olesen, K.: see *Heilskov, N. S. C.* 366 (T)
Olhagen, B. & *Svanborg, A.:* Attempts to prod experimental lipid nephrosis with tridione
Olsson, A.: see *Euler, U. S. von* 122

Paul, K. G.: Separation of porphyrins by me of counter-current distribution 212
Paulsen, L. & *Varnek, J.:* Rapid staining of sperm toza 295 (T)
Pers, M.: A method for the determination of glomerular filtration rate in patients with terocolic anastamosis 343
Poulsen, H.: see *Prætorius, E.* 273
Prætorius, E. & *Poulsen, H.:* Enzymatic det mination of uric acid 273.

Raaschou, F. & *Trautner, K.:* Obstruction of common bile duct in experimental renal hyp tension in dogs 223
Radner, S.: Method for recording aortic press pulses 129
Roholdt, K.: see *Milthers, K.* 102 (L)
Ryssing, E.: Influence of the surface tension serum and urine on the flame-photome analysis 321

Sandblom, Ph.: see *Euler, U. S. von* 122
Schroderus, K. A.: see *Timonen, S.* 207
Schönheyder, F.: see *Heilskov, N. S. C.* 366
Sommerfelt, S. Chr.: Comments regarding evaluation of paper electrophoresis of prot 105 (L)
Sommerfelt, S. Chr.: Paper electrophoresis isolated plasma protein fractions 299
Støa, K. F.: Determination of thiocyanate in bl serum 1
Sundberg, M.: Sensitized clotting time as a clin method 172
Svanborg, A.: see *Olhagen, B.* 146
Szücs, Zs.: see *Halmágyi, D.* 85

Cammelin, L.-E.: An electrometric method for the determination of cholinesterase activity I 267
Cammelin, L.-E.: see *Diamant, H.* 271
Csényi, M.: see *Halmágyi, D.* 85
Dhune, S.: see *Ekman, B.* 175
Eimonen, S. & Schroderus, K. A.: The effect of aneurin on uterine contractions 207
Frautner, K.: see *Raaschou, F.* 223
Fruedsson, E.: see *Ekman, B.* 175
Gunevall, G.: The antipneumolysin reaction and its clinical application 109
Varnek, J.: see *Paulsen, L.* 295 (T)
Vogt Lorentzen, F.: A simple method for the determination of functional residual air volume 132
Werner, S.: see *Heilskov, N. S. C.* 366 (T)
Zade-Oppen, M.: A method for determination of the osmotic resistance of erythrocytes by means of the electro-hemoscope 199 (L)
Zsóter, T.: see *Halmágyi, D.* 85
Åberg, B.: Breakdown of dextran by human feces 37

INDEX OF SUBJECTS

(L) = Letters to the Editors (T) = Technique and Practical Problems

Acidic compounds, in hydrolysed urine, with reference to disorders of the liver (Aterman, K., Boscott, R. J. & Cooke, W. T.) 296 (L)
ACTH, urinary calcium output during treatment with (Dragsted, P. J. & Hjorth, N.) 188
Adrenaline, in blood and urine by phaeochromocytoma (Euler, U. S. von, Lund, A., Olsson, A. & Sandblom, Ph.) 122
Agglutinogen skin test, in pertussis (Arvidson, M., Hedlund, P. & Mossberg, H.-O.) 44
Aneurin, effect on uterine contractions (Timonen, S. & Schroderus, K. A.) 207
Anion exchange resin, effect on pepsin (Hjort, P.) 350
Anticoagulant treatment, of cardiovascular disorders, with phenylindandione (Dedichen, J.) 324
Anticoagulant, Vitamin K as antagonist to (Hilden, T. & Munck, O.) 361
Antipneumolysin reaction, clinical application (Tunevall, G.) 109
Aortic pressure pulses, method for recording (Radner, S.) 129
Arterial pressure, pulmonary, effect of dihydroergotamine and hydergine on (Halmágyi, D., Iványi, J., Felkai, B., Zsóter, T., Tényi, M. & Szücs, Zs.) 85
Autoagglutinins, significance of (Bird, G. W. G.) 143

Base-binding power, of serum proteins (Broch, O. J.) 9
Benzylpenicillin-β-diethylaminoethylester hydriodide, inactivation in blood (Milthers, K. & Roholdt, K.) 102 (L)
Bile duct, obstruction of, in experimental renal hypertension in dogs (Raaschou, F. & Trautner, K.) 223
Biuret, micro method for protein determination (Goa, J.) 218
Blood, cholinesterase in (Tammelin, L.-E.) 267
Blood, effect of heparin and protamine sulphate on the occurrence of chylomicrons in (Grüner, A., Hilden, K. & Hilden, T.) 241
Blood, noradrenaline and adrenaline by phaechromocytoma (Euler, U. S. von, Lund, A. Olsson, A., & Sandblom, Ph.) 122
Blood, occurrence of chylomicrons in (Grüner, & Hilden, T.) 236
Blood spots, cholinesterase activity in (Heilbron E.) 308
Blood urea, in renal insufficiency (Haugen, H. & Blegen, E. M.) 63
Body fluids, base-binding power and function osmoregulators of serum proteins (Broch, J.) 9
Bone marrow, megaloblastic, effect of liver extra in cultures of (Nieweg, H. O.) 195
Brain tissue, cholinesterase in (Diamant, H. Tammelin, L.-E.) 271

Calcium, and magnesium, determination in ser (Grette, K.) 151
Calcium balance, phosphates and phosphoric a as dietary factors in (Malm, O. J.) 75
Calcium, direct titration in serum with ethyle diamine tetracetate (Lehmann, J.) 203 (L)
Calcium, urinary, output during treatment w ACTH (Dragsted, P. J. & Hjorth, N.) 188
Carbon dioxide, tension of end-tidal air (Lar T. K. I.) 90
Cardiovascular disorders, treatment with phen indandione (Dedichen, J.) 324
Cation exchange resin, failure to impair intesti absorption of terramycin (Grönroos, J.-A. Kalliomäki, L.) 378 (L)
Cerebrospinal fluid, determination of total prot in (Goa, J.) 218
Cholinesterase activity, electrometric determinati of (Tammelin, L.-E.) 267
Cholinesterase activity, electrometric determinati of (Diamant, H. & Tammelin, L.-E.) 271
Cholinesterase activity, electrometric determinati of (Heilbronn, E.) 308
Cholinesterase, in brain tissue (Diamant, H. Tammelin, L.-E.) 271
Cholinesterase, in human blood (Tammelin, L.-E 267

ıromatography, column, a new apparatus for (Kennedy, G. Y.) 201 (L)
ıromatography, of porphyrins (Kennedy, G. Y.) 281
ıromatography, paper, of porphyrin pigments (Eriksen, L.) 155
ıromatography, paper partition, of keto acids (Kulonen, E.) 72
ıylomicrons, occurrence in blood (Grüner, A. & Hilden, T.) 236
ıylomicrons, occurrence in blood, effect of heparin and protamine sulphate (Grüner, A., Hilden, K. & Hilden, T.) 241
earance, true endogenous creatinine (Haugen, H. N. & Blegen, E. M.) 67
otting time, sensitized, as a clinical method (Sundberg, M.) 172
olumn chromatography, a new apparatus for (Kennedy, G. Y.) 201 (L)
opper, serum, in normals (Brendstrup, P.) 312
opper-binding capacity, of blood serum (Brendstrup, P.) 18
ortisone, intraarticular injections, viscosity and hyaluronidase inhibitors in joint exudate after (Ekman, B., Thune, S. & Truedsson, E.) 175
ounter-current distribution, separation of porphyrins by means of (Paul, K. G.) 212
reatinine clearance, true endogenous (Haugen, H. N. & Blegen, E. M.) 67
reatinine, endogenous, in plasma and urine, determination of (Haugen, H. N.) 48
reatinine, endogenous plasma, in renal insufficiency (Haugen, H. N. & Blegen, E. M.) 63

extran, breakdown by human feces of (Åberg, B.) 37
icumarol, effect of Vitamin K on hypoprothrombinemia induced by (Hilden, T. & Munck, O.) 361
icumarol, therapy, prothrombin estimations during (Müllertz, S.) 179
ietary factors, phosphates and phosphoric acid in calcium balance (Malm, O. J.) 75
ihydroergotamine, effect on pulmonary arterial pressure in man (Halmágyi, D., Iványi, J., Felkai, B., Zsóter, T., Tényi, M. & Szücs, Zs.) 85

lectro-hemoscope, determination of osmotic resistance of erythrocytes by means of (Zade-Oppen, M.) 199 (L))
lectrometric determination, of cholinesterase activity (Tammelin, L.-E.) 267
lectrometric determination, of cholinesterase activity (Diamant, H. & Tammelin, L.-E.) 271
lectrometric method, for determination of cholinesterase activity (Heilbronn, E.) 308
lectrophoresis, paper, a photometric scanning device (Köiw, E.) 99 (L)
Electrophoresis, paper, of isolated plasma protein fractions (Sommerfelt, S. Chr.) 299
Electrophoresis, paper, of proteins, evaluation (Sommerfelt, S. Chr.) 105 (L)
Electrophoretic demonstration, of trypsin inhibitors in blood serum (Jacobsson, K.) 97 (L)
Electrophoretic investigations, of urine proteins in Masugi nephritis (Laurent, B. & Nikkilä, E. A.) 158
Embolism, artificial, peripheral, in the rabbit (Dencker, J. S.) 261
End-tidal air, tensions of oxygen and carbon dioxide in (Larmi, T. K. I.) 90
Enzymatic determination, of uric acid (Prætorius, E. & Poulsen, H.) 273
Erythrocytes, determination of osmotic resistance of, by means of electro-hemoscope (Zade-Oppen, M.) 199 (L)
Ethylenediamine tetracetate, determination of calcium and magnesium in serum with (Grette, K.) 151
Ethylenediamine tetracetate, direct titration of calcium in serum with (Lehmann, J.) 203 (L)

Fatty acids, saturated and unsaturated higher, determination in fecal fat (Kamer, J. H. van de) 30
Fecal fat, determination of saturated and unsaturated higher fatty acids in (Kamer, J. H. van de) 30
Feces, human, breakdown of dextran by (Åberg, B.) 37
Flame-photometric analysis, influence of serum and urine surface tension on (Ryssing, E.) 321
Flame photometry, internal standard, improved instrument for (Leppänen, V. & Hallman, N.) 250

Gas analysis, reagent for oxygen absorption (Devey, J. D.) 104 (L)
Globulin, β-, concentration in infantile leukemia (Brohult, A.) 328
Glomerular filtration rate, method for determination in patients with ureterocolic anastomosis (Pers, M.) 343
Glutamic acid, determination in urine by a modified Krebs' method (Foss, O. P.) 334
Glutamine, determination in urine by a modified Krebs' method (Foss, O. P.) 334
Hemolytic streptococci, typing of (Hedlund, P. & Lagercrantz, R.) 39
Heparin, effect on occurrence of chylomicrons in human blood (Grüner, A., Hilden, K. & Hilden, T.) 241
Heparin, synthetic substitutes, toxicity of (Astrup, T.) 137
Hepatic vein, occlusive, catheterization for estimation of portal venous pressure (Krook, H.) 285
Hepatitis, prothrombin estimations during (Müllertz, S.) 179

Hexamethonium, renal response to (Haugen, H. N. & Blegen, E. M.) 58
Hyaluronidase inhibitors, in joint exudate after intraarticular injection of cortisone (Ekman, B., Thune, S. & Truedsson, E.) 175
Hydergin, effect on pulmonary arterial pressure in man (Halmágyi, D., Iványi, J., Felkai, B., Zsóter, T., Tényi, M. & Szücs, Zs.) 85
Hypertension, experimental renal, in dogs (Raaschou, F. & Trautner, K.) 223
Hypoprothrombinemia, induced by dicoumarol, tromexan, and phenylindandione, effect of Vitamin K on (Hilden, T. & Munck, O.) 361

Internal standard flame photometry, improved instrument for (Leppänen, V. & Hallman, N.) 250
Intestinal absorption, of terramycin (Grönroos, J.-A. & Kalliomäki, L.) 378 (L)
Iron, serum concentration, diurnal variation (Laurell, C.-B.) 118
Iron, serum, in normals (Brendstrup, P.) 312
Iron-binding capacity, of serum in normals (Brendstrup, P.) 312
Isotopes, nitrogen, mass spectrometer (Heilskov, N. S. C., Olesen, K., Schønheyder, F. & Werner, S.) 366 (T)

Joint exudate, viscosity and hyaluronidase inhibitors after cortisone injections (Ekman, B., Thune, S. & Truedsson, E.) 175

Keto acids, paper partition chromatography of (Kulonen, E.) 72
Krebs' method, for determination of glutamine and glutamic acid in urine, modified (Foss, O. P.) 334

Leukemia, infantile, β-globuline concentration and treatment of (Brohult, A.) 328
Lipid nephrosis, experimental, with tridione (Olhagen, B. & Svanborg, A.) 146
Liver, acidic and phenolic compounds of urine by disorders of (Aterman, K., Boscott, R. J. & Coke, W. T.) 296 (L)
Liver extract, effect in cultures of megaloblastic bone narrow (Nieweg, H. O.) 195

Magnesium, and calcium, determination in serum (Grette, K.) 151
Mass spectrometer, in analytical work with nitrogen isotopes (Heilskov, N. S. C., Olesen, K., Schønheyder, F. & Werner, S.) 366 (T)
Masugi nephritis, urine proteins in (Laurent, B. & Nikkilä, E. A.) 158
Megaloblastic bone marrow, effect of liver extract in cultures of (Nieweg, H. O.) 195
Metabolites, abnormal, in the urine of phenylketonuria (Boscott, J. R. & Bickel, H.) 380 (L)

Nephritis, Masugi, urine proteins in (Laurent, & Nikkilä, E. A.) 158
Nephrosis, lipid, experimental, with tridione (hagen, B. & Svanborg, A.) 146
Nitrogen, isotopes, mass spectrometer (Heilsk N. S. C., Olesen, K., Schønheyder, F. & W ner, S.) 366 (T)
Noradrenaline, in blood and urine, by phaeochro cytoma (Euler, U. S. von, Lund, A., Ols A. & Sandblom, Ph.) 122

Osmoregulators in body fluids, function of se proteins as (Broch, O. J.) 9
Osmotic resistance, of erythrocytes, determina by means of electro-hemoscope (Zade-Op M.) 199 (L)
Ox plasma, prothrombinfree, preparation for mation of prothrombin (Brodthagen, 376 (T)
Oxygen, absorption in gas analysis, reagent (Devey, J. D.) 104 (L)
Oxygen, and carbon dioxide, tensions of, in tidal air (Larmi, T. K. I.) 90

Paper chromatography, of porphyrin pigm (Eriksen, L.) 155
Paper electrophoresis, a photometric scan device (Köiv, E.) 99 (L)
Paper electrophoresis, of isolated plasma pro fractions (Sommerfelt, S. Chr.) 299
Paper electrophoresis, of proteins, evaluation (S merfelt, S. Chr.) 105 (L)
Paper partition chromatography, of keto a (Kulonen, E.) 72
Penicillin, benzyl-β-diethylaminoethylester hyd dide, inactivation in blood (Milthers, K. Roholdt, K.) 102 (L)
Pepsin, effect of anion exchange resin on (Hj P.) 350
Pertussis, agglutinogen test in (Arvidson, M., H lund, P. & Mossberg, H.-O.) 44
Phaeochromocytoma, noradrenaline and adrena in blood and urine by (Euler, U. S. von, L A., Olsson, A. & Sandblom, Ph.) 122
Phenolic compounds, in hydrolysed urine, reference to disorders of the liver (Aterman, Boscott, R. J. & Cooke, W. T.) 296 (L)
Phenylindanedione, effect of Vitamin K on hy prothrombinemia induced by (Hilden, T. Munck, O.) 361
Phenylindanedione, treatment of cardiovasc disorders with (Dedichen, J.) 324
Phenylketonuria, abnormal metabolites in urine (Boscott, R. J. & Bickel, H.) 380 (L)
Phosphates, as dietary factor in calcium bala (Malm, O. J.) 75
Phosphoric acid, as dietary factor in calc balance (Malm, O. J.) 75
Photoelectric micro method, for direct titration calcium in serum, with ethylenediamine te cetate (Lehman, J.) 203 (L)

hotometric scanning device, for paper electrophoresis (Köiw, E.) 99 (L)
hotometry, flame, internal standard, improved instrument for (Leppänen, V. & Hallman, N.) 250
ID-trombantin, treatment of cardiovascular disorders with (Dedichen, J.) 324
ipettes, arrangement for cleaning of (Lundin, J.) 293 (T)
lasma, determination of endogenous creatinine in (Haugen, H. N.) 48
lasma, endogenous creatinine of, in renal insufficiency (Haugen, H. N. & Blegen, E. M.) 63
lasma, ox, prothrombin-free, preparation of, for estimation of prothrombin (Brodthagen, H.) 376 (T)
lasma protein fractions, isolated, paper electrophoresis of (Sommerfelt, S. Chr.) 299
orphyrin pigments, paper chromatography of (Eriksen, L.) 155
orphyrins, partition chromatography of (Kennedy, G. Y.) 281
orphyrins, separation by means of counter-current distribution (Paul, K. G.) 212
ortal venous pressure, estimation by occlusive hepatic vein catheterization (Krook, H.) 285
ressure recordings, in upper urinary tract (Kiil, F.) 383 (L)
rotamine sulphate, effect on occurrence of chylomicrons in human blood (Grüner, A., Hilden, K. & Hilden, T.) 241
rotein, determination, micro biuret method for (Goa, J.) 218
rotein, isolated plasma fractions, paper electrophoresis of (Sommerfelt, S. Chr.) 299
roteins, paper electrophoresis of (Sommerfelt, S. Chr.) 105 (L)
roteins, urine, in Masugi nephritis (Laurent, B. & Nikkilä, E. A.) 158
roteinuria, experimental, studies on (Laurent, B. & Nikkilä, E. A.) 158
rothrombin, estimations during hepatitis and dicoumarol therapy (Müllertz, S.) 179
rothrombin, estimation with prothrombin-free ox plasma (Brodthagen, H.) 376 (T)
ulmonary arterial pressure, effect of dihydroergotamine and hydergine on (Halmágyi, D., Iványi, J., Felkai, B., Zsóter, T., Tényi, M. & Szücs, Zs.) 85
ulses, aortic, method for recording (Radner, S.) 129

enal hypertension, experimental, in dogs (Raaschou, F. & Trautner, K.) 223
enal insufficiency, blood urea and plasma endogenous creatinine in (Haugen, H. N. & Blegen, E. M.) 63
enal response, to hexamethonium (Haugen, H. N. & Blegen, E. M.) 58
esidual air, functional, method for determination of (Vogt Lorentzen, F.) 132
Resin, anion exchange, effect of pepsin on (Hjort, P.) 350
Resin, cation exchange, failure to impair intestinal absorption of terramycin (Grönroos, J.-A. & Kalliomäki, L.) 378 (L)

Scarlet fever, typing of hemolytic streptococci in (Hedlund, P. & Lagerkrantz, R.) 39
Serum copper, in normals (Brendstrup, P.) 312
Serum, copperbinding capacity of (Brendstrup, P.) 18
Serum, determination of calcium and magnesium Grette, K.) 151
Serum, determination of thiocyanate in (Støa, K. F.) 1
Serum, direct titration of calcium with ethylenediamine tetracetate (Lehman, J.) 203 (L)
Serum, electrophoretic demonstration of trypsin inhibitors in (Jacobsson, K.) 97 (L)
Serum iron, concentration, diurnal variation of (Laurell, C.-B.) 118
Serum iron, in normals (Brendstrup, P.) 312
Serum, iron-binding capacity in normals of (Brendstrup, P.) 312
Serum proteins, base-binding power of, and function as osmoregulators in body fluids (Broch, O. J.) 9
Serum, surface tension in, influence on flame-photometric analysis (Ryssing, E.) 321
Skin test, agglutinogen, in pertussis (Arvidson, M., Hedlund, P. & Mossberg, H.-O.) 44
Spectrometer, mass, in analytical work with nitrogen isotopes (Heilskov, N. S. C., Olesen, K., Schønheyder, F. & Werner, S.) 366 (T)
Spermatozoa, rapid staining of (Paulsen, L. & Varnek, J.) 295 (T)
Staining, of spermatozoa (Paulsen, L. & Varnek, J.) 295 (T)
Streptococci, hemolytic, typing of (Hedlund, P. & Lagerkrantz, R.) 39
Surface tension, in serum and urine, influence on flame-photometric analysis (Ryssing, E.) 321

Tbc. laboratories, precautions against infection of personnel (Engbæk, H. Chr.) 198 (T)
Terramycin, intestinal absorption of (Grönroos, J.-A. & Kalliomäki, L.) 378 (L)
Thiocyanate, in serum, determination of (Støa, K. F.) 1
Toxicity, of synthetic heparin substitutes (Astrup, T.) 137
Tridione, experimental lipid nephrosis with (Olhagen, B. & Svanborg, A.) 146
Tromexan, effect of Vitamin K on hypoprothrombinemia induced by (Hilden, T. & Munch, O.) 361
Trypsin inhibitors, electrophoretic demonstration of (Jacobsson, K.) 97 (L)

Urea, blood, in renal insufficiency (Haugen, H. N. & Blegen, E. M.) 63

Ureterocolic anastomosis, determination of glomerular filtration rate in patients with (Pers, M.) 343
Uric acid, enzymatic determination (Prætorius, E. & Poulsen, H.) 273
Urinary calcium, output during treatment with ACTH (Dragsted, P. J. & Hjorth, N.) 188
Urinary tract, pressure recordings in (Kiil, F.) 383 (L)
Urine, determination of endogenous creatinine in (Haugen, H. N.) 48
Urine, determination of glutamine and glutamic acid by a modified Krebs' method (Foss, O. P.) 334
Urine, hydrolysed, acidic and phenolic compounds, with reference to liver disorders (Aterman, K., Boscott ,R. J. & Cooke, W. T.) 296 (L)
Urine, noradrenaline and adrenaline by phaeochromocytoma (Euler, U. S. von, Lund, A., Olsson, A. & Sandblom, Ph.) 122
Urine, of phenylketonuria, abnormal metabolites (Boscott, R. J. & Bickel, H.) 380 (L)
Urine, proteins, in Masugi nephritis (Laurent, & Nikkilä, E. A.) 158
Urine, surface tension in, influence on flame-pho metric analysis (Ryssing, E.) 321
Uterine contractions, effect of aneurin on (Tir nen, S. & Schroderus, K. A.) 207

Ventilatory insufficiency, oxygen and carbon diox tensions of end-tidal air in (Larmi, T. K. I.)
Viscosity in joint exudate, after intraarticular c tisone injections (Ekman, B., Thune, S. Truedsson, E.) 175
Vitamin K, as antagonist to anticoagulant (Hild T. & Munck, O.) 361
Vitamin K, effect in hypoprothrombinemia indu by dicoumarol, tromexan and phenylindanedi (Hilden, T. & Munck, O.) 361

CORRIGENDUM

Vol. 5, p. 151, 1953: *Micromethod for the determination of calcium and magnesium in serum.*
P. 153, bottom line:

$$= \frac{\text{Titration in ml}}{\text{Volume of Ca-standard (ml)}} \times 5$$

correct to:

$$= \frac{\text{Volume of Ca-standard (ml)}}{\text{Titration in ml}} \times 5$$

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CONTENTS

S. CHR. SOMMERFELT: Paper electrophoresis of isolated plasma protein fractions 299
E. HEILBRONN: An electrometric method for the determination of cholinesterase activity 308
P. BRENDSTRUP: Serum iron, total iron-binding capacity of serum and serum copper in normals 312
E. RYSSING: Influence of the surface tension in serum and urine on the flame-photometric analysis 321
J. DEDICHEN: Long term anticoagulant treatment of cardiovascular disorders with phenylindandione (pid-trombantin) 324
A. BROHULT: β-globulin concentration and treatment of infantile leukemia 328
O. P. FOSS: On the determination of glutamine and glutamic acid in urine by a modification of Krebs' method 334
M. PERS: A method for the determination of the glomerular filtration rate in patiens with ureterocolic anastomosis 343
P. HJORT: The effect of an anion exchange resin on pepsin 350
T. HILDEN AND O. MUNCH: Vitamin K as antagonist to anticoagulants 361

Technique and practical problems

N. S. C. HEILSKOV, K. OLESEN, F. SCHØNHEYDER AND S. WERNER: Experiences with a mass spectrometer in analytical work with nitrogen isotopes 366
H. BRODTHAGEN: The preparation of prothrombin-free ox plasma in the estimation of prothrombin 376

Letters to the editors

J. A. GRÖNROOS AND L. KALLIOMÄKI: Failure of cation exchange resin to impair intestinal absorption of terramycin 378
R. J. BOSCOTT AND H. BICKEL: Detection of some new abnormal metabolites in the urine of phenylketonuria 380
F. KIIL: Pressure recordings in the upper urinary tract 383

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